Studies of Golgi organization and protein secretion in yeasts

Thesis for the degree of
Doctor of Philosophy

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Thanks to Mum and Dad

for love and support

Declaration

The work presented in this thesis was carried out under the supervision of Dr. Alan Boyd at the Department of Biochemistry, University of Edinburgh and by Dr. Dave Pioli at Zeneca Pharmaceuticals, Alderly Park between November 1990 and December 1994.

The experimental work presented in this thesis, unless stated otherwise, is my own and this manuscript has been composed by myself

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Abstract

The *PMR1* gene of *Saccharomyces cereivisiae* is predicted to encode a P-type Ca^{2+} ATPase (Rudolph *et al.*, 1989). This protein has been reported as localizing to a novel Golgi-like organelle (Antebi and Fink, 1992). Consistent with Pmr1p's proposed Golgi distribution is the fact that the pleiotropic phenotype of null mutants results in defects in various Golgi processes (*e.g.* outer chain glycosylation, proteolytic processing, vacuolar sorting). These defects are reversible by addition of Ca^{2+} to the extracellular medium, supporting the proposed function as a Ca^{2+} pump (Rudolph *et al.*, 1989; Antebi and Fink, 1992). Work described in this thesis was carried out to investigate the nature of the *pmr1* phenotype and to further characterize the Pmr1p containing organelle(s). It is demonstrated that the *pmr1* phenotype is not due to a complete bypass of the Golgi (as proposed by Rudolph *et al.*, 1989) since Kex2p processing of a secreted protein is detected in a *pmr1* mutant. There does however appear to be a change in Golgi organization in *pmr1* mutants. When the organelles containing Kex2p were isolated from *pmr1* and *PMR1* strains the profiles of other marker enzymes recovered was significantly altered. In particular the enzyme GDPase, an early Golgi marker, colocalizes with Kex2p, a *trans*-Golgi network marker, in *pmr1* strains. Furthermore DPAP A (Ste13p) no longer colocalizes with Kex2p in *pmr1* strains. This evidence suggests that protein targeting and/or retention is altered in *pmr1* strains as a result of aberrant Ca^{2+} homeostasis. Another possibility is that some gross reorganization of Golgi structure has occurred. These changes in enzyme localization were not reversible by addition of Ca^{2+} to the growth media.

Pmr1p was tagged with protein A to allow isolation of organelles containing the fusion protein with IgG-Sepharose. However, after characterization of the recovered material it became clear that the fusion protein had localized to the vacuole and not to the Golgi. This is probably due to the tag interfering with proper Golgi retention This result is consistent with the vacuolar default model for membrane proteins in yeast (Roberts *et al.*, 1992; Nothwher *et al.*, 1993).

The vacuolar default model suggests that yeast plasma membrane proteins are likely to contain targeting signals. To test this the plasma membrane protein Pma1p was fused to a Kex2p-protein A fusion protein which localizes to the vacuole. The tribrid protein localized to the Golgi and not the plasma membrane suggesting either fusion of Pma1p has restored Kex2p retention in the Golgi, or that the secretory pathway is simply unable to cope with such an unusual fusion protein.

The final piece of work in this thesis involves a preliminary study of the usefulness of the yeast *Kluyveromyces lactis* as a host for the secretion of heterologous proteins. In the test case described biologically-active human elafin, an elastase inhibitor, is secreted at levels 8 time higher than from a *S. cerevisiae* strain.

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Abbreviations used in this thesis

ATP	adenosine 5'-triphosphate
bp	base pair
CHO	Chinese hamster ovary
COP	coat protein
CPY	carboxypeptidase Y
Da	dalton
DNA	deoxyribonucleic acid
DPAP	dipeptidyl aminopeptidase
ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	diaminoethanetetra-acetic acid
EGTA	1,2-di(2-aminoethoxy) ethane-N,N,N',N'-tetra acetic acid
ER	endoplasmic reticulum
g	relative centrifugal force
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRP	horse radish peroxidase
Ig	immunoglobulin
ImAd	immunoadsorbant
K	thousand
Kb	thousand bases
K. lactis	*Kluyveromyces lactis*
mg	milligram

Abbreviations (continued)

min	minute
ml	millilitre
µg	microgram
µl	microlitre
NEM	N-ethylmaleimide
nm	nanometres
NSF	NEM-sensitive factor
^{o}C	degrees centigrade
OD_{600}	optical density at 600 nm
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
Spa	*Staphylococcal* protein A
TCA	trichloroacetic acid
TGN	*trans* Golgi network
Tris	2-amino-2-(hydroxymethyl) propane-1,3-dio(tris)
VSV	vesicular stomatitus virus

Abbreviations for amino acids

Amino Acid	Abbreviations	
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamate	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Therinine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Chapter 1
Introduction

1 The compartmental organization of eukaryotic cells

Eukaryotic cells have evolved compartments or organelles which maintain distinct subcellular environments allowing each reaction within a cell to occur in optimal conditions (for a general overview of eukaryotic cell structure see Alberts *et al.*, 1994). Maintenance of these environments is essential for the efficient running of the cell and this relies upon the accurate distribution of proteins and lipids to the various organelles. Eukaryotic cells have evolved a secretory pathway which facilitates the distribution of proteins and lipids to the various organelles as well as allowing secretion of molecules into the extracellular environment. The secretory pathway has been defined in mammalian cells (reviewed by Palade, 1975) and in the yeast *Saccharomyces cerevisiae* (in this thesis yeast refers to *S. cerevisiae*) . *S. cerevisiae* is a simple eukaryotic cell which is easily genetically manipulated and analysis of its secretory pathway has revealed a great deal about how proteins are modified, targeted, and retained. A combination of work on yeast and animal cell-free systems has revealed many details of the molecular machinery of the secretory pathway (reviewed by Rothman and Orci, 1992; Pryer *et al.*, 1992) and shown that many of these mechanisms are conserved in evolution.

The early work on the secretory pathway in mammalian cells involved following radioactive molecules through the secretory pathway in pulse-chase experiments (Palade, 1975). Progress of these molecules could be monitored using the then recently improved subcellular fractionation techniques and electron microscopy. In yeast the identification of mutants in the secretory pathway allowed the sequence of post-translational events in the secretion of glycoproteins to be defined confirming observations made in mammalian cells. Novick *et al.* (1980) identified mutant *S. cerevisiae* cells which were temperature-sensitive for secretion and cell surface growth and became dense at the non-permissive temperature (37^{o}C). This increase in

density was due to the accumulation of various organelles within the mutants. This was the basis of their screen which identified 23 complementation groups required for post-translational events in the secretory pathway. They went on to order these *sec* mutants within the secretory pathway by using pairwise combinations of mutations to observe the effect on the structure of accumulated invertase and the morphology of the exaggerated organelles (Novick *et al.*, 1981).

It is now known that in all eukaryotes a protein's entry into the secretory pathway involves translocation across, or insertion into, the membrane of the endoplasmic reticulum (ER). This is followed by transport in vesicles which bud from the ER and are targeted to the Golgi network. Here the vesicles fuse with a specific target membrane allowing the proteins they carry to continue through the pathway. Transport through the Golgi network is also mediated by vesicular transport and as proteins pass through the various compartments they are modified and sorted. The destination of a protein depends upon what, if any, targeting or retention signal it carries. Pathways to the plasma membrane, lysosome (or vacuole in yeast) branch at the Golgi and specific targeting signals ensure the correct proteins enter budding vesicles destined for the correct target membranes.

The mammalian and yeast secretory pathways are broadly similar but several differences of note do exist. The following section will describe the passage of a protein through a secretory pathway highlighting both the differences and similarities in *S. cerevisiae* and mammalian cells (**figure 1-1**). Proteins are targeted to the secretory pathway by specific N-terminal signal sequences consisting of a stretch of 15-30, mainly hydrophobic, amino acids (von Heijine G, 1985). These signal sequences target the protein to the ER membrane across which the polypeptide is translocated. For most proteins destined to be secreted, or reside as soluble luminal

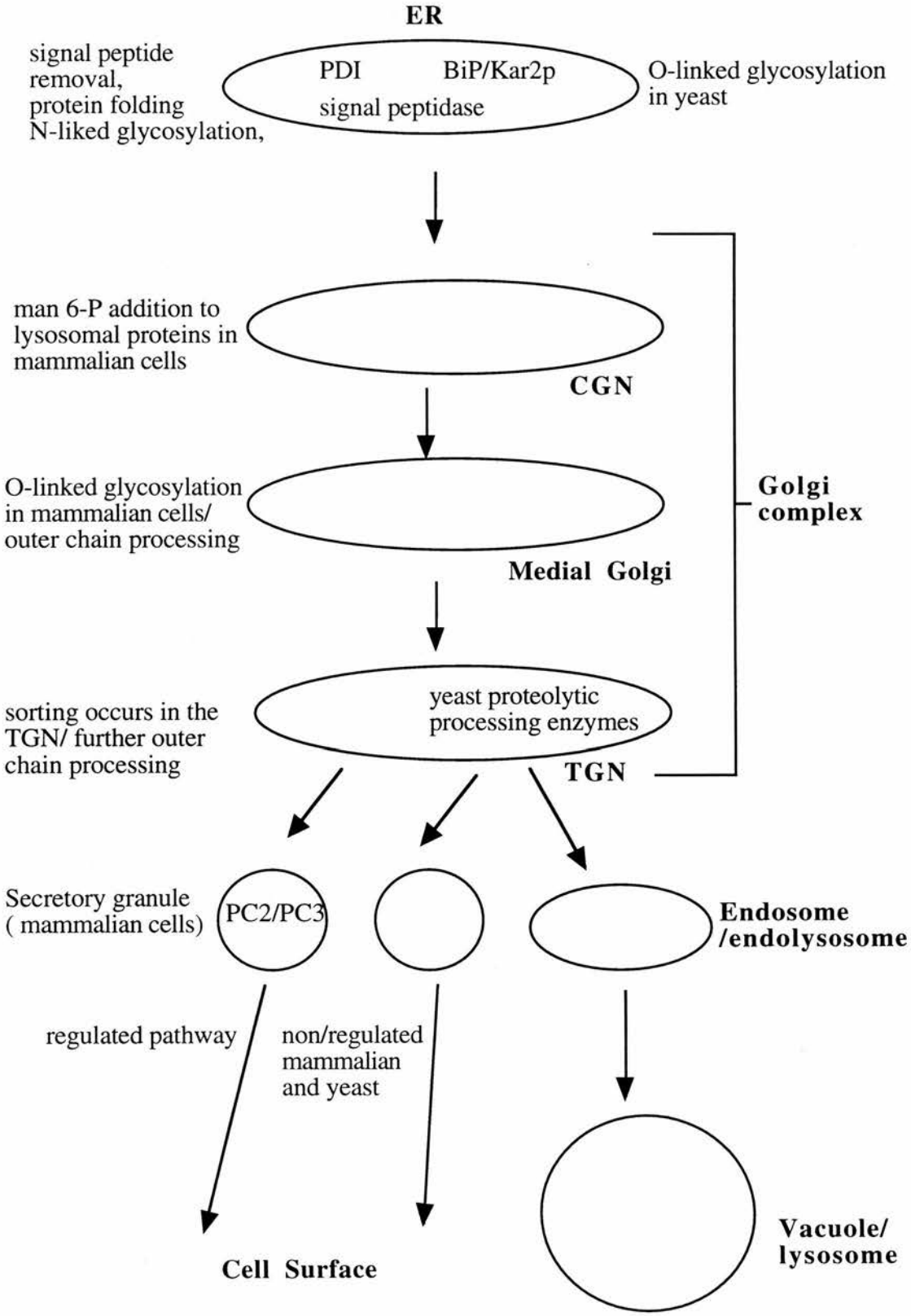


Figure 1-1 A summary of the secretory pathway in yeast and mammalian cells

proteins at some point in the pathway, the signal sequence is cleaved off once the polypeptide enters the ER lumen. This cleavage is performed by a signal peptidase complex which was first identified in canine pancreatic rough microsomes (Evans *et al.*, 1986) and consists of four proteins. In yeast the essential *SEC11* gene encodes a protein required for signal peptidase processing which has significant identity to two of the mammalian polypeptides found in the signal peptidase complex. In membrane proteins the signal sequence is often utilized as a transmembrane sequence and is not cleaved.

Mammalian cell-free assay systems which reproduced targeting and translocation of proteins across the ER membrane led to the isolation of a signal recognition particle (SRP) (Walter and Blobel, 1980) and its receptor (Gilmore *et al.*, 1982a, 1982b). Mammalian SRP is a 11S cytoplasmic ribonucleoprotein consisting of six polypeptides and a 7SL RNA molecule. The SRP binds to the signal peptide as soon as the peptide emerges from the ribosome causing a pause in protein synthesis which allows for docking with the ER membrane. Translation begins again once the SRP/ribosome complex binds to the SRP receptor found in the rough ER. A yeast homologue of this receptor has been identified (*SRP101*) (Ogg *et al.*, 1992)

Translocated proteins are folded up in the lumen of the ER. Unfolded polypeptides in the ER lumen are prevented from precipitating by the binding of the binding protein BiP (Kar2p in yeast) (Gething and Sambrook, 1992; Normington *et al.*, 1989). BiP binds ATP and is structurally related to the hsp70 family of stress-response proteins. These proteins are important to help solubilize and refold proteins that have been damaged by heat shock or other stresses (Pelham, 1986; Ellis and Van der Vies, 1991). Associated with the luminal side of the ER membrane is an enzyme, protein disulphide isomerase (PDI) which catalyzes the formation of disulphide bonds,

important in the folding and structure of proteins (Freedman, 1984).

Proteins are also glycosylated as they enter the lumen of the ER. Oligosaccharide chains are added to selected asparigine residues (Asn-X-Ser or Asn-X-Thr sequences, where X is any amino acid except proline). A core oligosaccharide chain built up from three glucose, nine mannose and two N-acetyl glucosamine molecules ($Man_9Glc_3GlcNAc_2$) is transferred from the lipid donor dolichol to the amide nitrogen of an asparagine residue. Further processing of this $Man_9Glc_3GlcNAc_2$ moiety occurs in the ER before transport to the Golgi. In yeast cells glucose residues and a single α-1,2 mannose residue are removed by glucosidases and a mannosidase respectively. The resulting $Man_8GlcGlcNAc_2$ moiety is then extended by the addition of six mannose residues in a reaction catalysed by a mannosyl transferase (Kukuruzinska *et al.*, 1987). In mammalian cells similar processing occurs with a glucose and a α-1,2 mannose residue being removed to form a high mannose oligosaccharide, typical of mammalian glycoproteins. In yeast the ER is also the site of O-linked glycosylation. Certain serine and threonine residues found in proteins are the target for attachment of oligosaccharides (Haselbeck and Tanner, 1983), although no consensus target sequence has been identified. In mammalian cells O-linked glycosylation is initiated in the Golgi apparatus and not in the ER (Niemann *et al.*, 1982; Johnson and Spear, 1983).

Once proteins have correctly folded and have been modified in the ER as described above they are packaged into vesicles and transported to the Golgi apparatus. Here the oligosaccharides added in the ER are further modified. In mammalian cells some of the high mannose chains formed in the ER are processed to complex oligosaccharides which may contain additional GlcNAc residues as well as variable numbers of galactose, sialic acid and fucose residues (Kornfeld and Kornfeld, 1985). N-linked oligosaccharide chains added in yeast ER are also modified in the Golgi, being

extended by the addition of many mannose residues to form an outer chain structure which may contain more than fifty residues.

The Golgi apparatus of mammalian cells and yeast cells carry out other similar processes. As well as modifying oligosaccharides the Golgi is where sorting of proteins to various destinations in the cell is performed. The trans-Golgi-network (TGN) is where proteins destined for lysosomes (mammalian cells), vacuoles (yeast), secretory granules (mammalian cells), and the plasma membrane are sorted into specific vesicles. Soluble lysosomal hydrolases are targeted by addition of a mannose 6-phosphate (man 6-P) residue to their oligosaccharide side chain (Kornfeld, 1986). Proteins which acquire this man 6-P residue are sorted in the TGN into vesicles destined for the endolysosome. Two related transmembrane proteins are receptors for the man 6-P residue and concentrate lysosomal proteins into clathrin coated pit areas which form vesicles destined for the endolysosome (Brown *et al.*, 1986; Duncan and Kornfeld, 1988). On fusion with the endolysosome the receptor molecules are recycled back to the TGN. In yeast sorting of soluble proteins to the vacuole has not been shown to be dependent on man 6-P. A targeting sequence for yeast carboxypeptidase Y (CPY) has been identified within its primary sequence as QRPL (Valls *et al.*, 1987) and a TGN receptor for this sequence also identified (Marcusson *et al.*, 1994)

Both yeast and mammalian secretory pathways contain several proteases which function to process various pro-proteins. In yeast Kex2p, DPAP A, and Kex1p are found in the late Golgi where they process pro-α-factor (see section **3-1**). Certain mammalian cells have a regulated secretory pathway where secretory proteins and other molecules are stored before release upon stimulation. Yeast have no similar pathway. PC2 and PC3 are Ca^{2+}-dependent serine proteases structurally related to the

yeast Kex2p. Both are expressed specifically in neuroendocrine tissues where they function within granules associated with the regulated secretory pathway. Here they process precursors such as proinsulin and propoiomelanocortin (Smoekens *at al.*, 1992; Thomas *et al.*, 1991).

2 ER protein retention

2-1 Is protein export from the ER unselective or selective?

Proteins destined to enter the secretory pathway carry signal peptides which target them to the ER membrane. Once targeted they are co-translationally translocated across the ER membrane (reviewed by Simon, 1993; Ng and Walter, 1994). Membrane proteins contain one or more stop translocation signals which form the transmembrane sequences. Early opinion favoured the idea that transport of proteins out of the ER occurs by default (Weiland *et al.*, 1987; Pfeffer and Rothman, 1987) and two models were proposed to explain export of some proteins and retention of others in the ER. The first was that export was selective and that an unselective retention mechanism existed. This would mean exported proteins would possess a signal to mark them for transport. The second model was that retention was selective and export unselective. It follows that retained proteins would carry a retention signal.

The two models were distinguished by measuring the half-time for secretion of inert synthetic tripeptides (Asn-Tyr-Thr/Ser) from HepG2 and CHO cells (Weiland *et al.*, 1987). These tripeptides could permeate membranes but once inside the ER they were N- glycosylated preventing exit. If protein transport was selective then the synthetic tripeptides should be secreted with a much slower half-time than proteins. If retention was selective then the tripeptides should be secreted as fast as or faster than proteins. The latter proved correct and the selective retention model was substantiated. The results also indicated that no signal was required for transport to the plasma membrane

and by implication that signals must exist to deviate a protein from this default pathway.

More recent work in yeast casts some doubt on the results presented by Weiland *et al.* (1987). Romisch and Schekman (1992) demonstrated that the tripeptide Asn-Tyr-Thr could exit the ER under conditions which inhibit vesicle formation and the exit of the proα-factor protein. Furthermore the core glycosylated tripeptide was not modified by Golgi processing enzymes. Release of the tripeptide was shown to be dependent on ATP and Romisch and Schekman (1992) proposed that an ATP-dependent pump in the ER membrane might be responsible for export.

2-2 Retention of soluble ER proteins

Retention signals within the primary sequence of ER soluble and membrane proteins have been identified (reviewed by Pelham, 1989a; Pryer *et al.*, 1992). In mammalian cells the C-terminal tetrapeptide KDEL (-lysine-aspartate-glutamate-leucine) serves as the signal for retention of soluble ER proteins and confers ER localization to proteins destined for the cell surface (Munro and Pelham, 1987). In yeast the equivalent sequence was found to be HDEL (-histidine-aspartate-glutamate-leucine) (Pelham *et al.*, 1988).

An obvious explanation for the retention of ER proteins would be for a receptor to exist in the ER which specifically binds to proteins carrying KDEL or HDEL. However no candidates exist for a receptor of sufficient abundance in the ER to stoichiometrically bind all the retained proteins. Munro and Pelham (1987) therefore proposed a retrieval mechanism whereby proteins escaping from the ER were continuously retrieved from a subsequent compartment. Strong evidence for this came from experiments which showed that proteins tagged with KDEL showed signs of

having certain Golgi modifications (Pelham 1988). A salvage compartment was proposed to exist at the *cis*-side of the Golgi network where retrieval took place (Lazzarino and Gabel, 1988; Pelham, 1989b).

The retention system for ER soluble proteins showed all the signs of being a receptor-mediated process. Depending on the cell type, only HDEL or KDEL was efficiently recognised, suggesting a highly specific mechanism. In addition retention was saturable since overexpression of HDEL-tagged proteins in yeast disrupted retention of resident ER HDEL containing proteins. The HDEL receptor was identified in yeast by genetic means and is the product of the *ERD2* gene (Lewis *et al.*, 1990; Smenza *et al.*, 1990). A mammalian *ERD2* homologue has been identified by PCR (Lewis and Pelham, 1990).

Optimal binding to ligands by Erd2p occurs at acidic pH (Wilson *et al.*, 1993) and a model has been proposed whereby Erd2p binds KDEL or HDEL in an early Golgi or pre-Golgi salvage compartment. The result is recycling of proteins back to the ER. In the higher pH environment of the ER the proteins are released and Erd2p returns to the salvage compartment. Supporting this model human Erd2p, when expressed in COS cells, is normally found in or near the Golgi apparatus but high level expression of appropriate ligands, such as KDEL tagged lysozyme, causes a shift towards the ER (Lewis and Pelham, 1992; Townsly *et al.*, 1993).

2-3 Retention of ER membrane proteins

Retention signals have been identified in several ER-resident membrane proteins. Although Sec20p, in yeast, has been shown to have an HDEL sequence (Sweet and Pelham, 1992) in general ER-membrane proteins possess retention signals consisting of either two critical lysines (K) or arginines (R). Type I membrane proteins have

signals containing lysine (Nilsson *et al.*, 1989; Jackson *et al.*, 1990; Jackson *et al.*, 1993) and type II membrane proteins have signals containing arginines (Shultz *et al.*, 1994). Mutational analysis of these signals showed that if these critical residues were replaced by other amino acids the proteins would leave the ER and pass through the secretory pathway to the plasma membrane, supporting the idea of a default pathway to the plasma membrane for membrane proteins in mammalian cells. Recently the role of the dilysine motif has been examined in yeast ER membrane protein retention. The ER membrane protein Wbp1p has a dilysine motif (Gaynor *et al.*, 1994) and invertase can be retained in the ER by fusing it to the Wbp1p sequence containing the transmembrane domain and dilysine motif. Mutations in this motif result in the fusion protein's delivery to the vacuole (consistent with the vacuolar default model, see section **3-3**). Hill and Stevens (1994) have shown that the ER membrane protein Vma1p is also retained by a dilysine motif.

2-4 Retention or retrieval?

The best evidence that double-LL-, double-RR-, -HDEL, and -KDEL sequences direct retrieval of proteins to the ER is that demonstrating Golgi modification of these proteins. However most ER proteins do not show these Golgi modifications (Jackson *et al.*, 1993). Furthermore removal of ER targeting motifs from various endogenous ER proteins, such as BiP (Munro and Pelham, 1987) and ERp59/PDI (Mazzarella *et al.*, 1990) results at best in slow transport of these molecules out of the ER. These observations suggest that most ER proteins never leave and are prevented from doing so by some mechanism other than the motifs discussed above. The combination of this retention mechanism and the retrieval system, which does rely on these motifs, ensures minimal leakage from the ER and this is illustrated by recent studies on the soluble resident ER protein calreticulin (Sonnichsen *et al.*, 1994). Calreticulin (CR) has a high Ca^{2+}-binding capacity (Michalak *et al.*, 1992), a

C-terminal KDEL motif and is glycosylated in the Golgi. Sonnichsen *et al* (1994) cloned the rat CR gene and overexpressed it in COS cells. It was predicted that there could be a redistribution of CR to the Golgi, and significant secreted levels would be detected due to saturation of the retrieval system, but immunolocalization and subcellular fractionation showed this was not the case, since most CR still had an ER distribution. This suggested that CR must leave the ER at a slow rate, and/or the retrieval system was very quick and efficient. The Erd2p KDEL receptor did not redistribute to the ER as expected if CR was significantly retained by the retrieval system. EM studies showed that the ER was dilated presumably to accommodate up to 100 times wild type levels of CR. Mutation of KDEL to HVEL or complete removal of KDEL had little effect on CR localization; clearly some other mechanism was involved in CR localization. The Ca^{2+}-binding domain (C-domain) of CR was proposed as a putative additional retention domain. This was based on evidence that addition of Ca^{2+} chelators to murine fibroblasts results in the secretion of protein disulphide isomerase (PDI) (Booth and Koch, 1989). Removal of the C-domain resulted in complete redistribution of the Erd2p receptor and significant secretion of the mutant CR. The retrieval system on its own is obviously unable to cope with the extremely high levels of CR. Possible mechanisms for non-retrieval ER retention are the formation of protein oligomers or a matrix in the lumen. Many proteins are known to form oligomers (Hurtley and Helenius, 1989) and it has been suggested that oligomeric structures could form that are large enough to exclude proteins from transport vesicles. Much evidence for this sort of retention mechanism comes from study of Golgi protein retention which is discussed in section **3-2**. The question arises why do some proteins apparently cycle between the ER and an early Golgi compartment? It may be that proteins of great importance to ER function have a motif to allow retrieval so that in times of stress retention is maximised. Another possibility is that these proteins are required to carry out their function in more than one

compartment.

3 Golgi structure, protein retention and sorting

3-1 Golgi organization and morphology

The compartments of the mammalian Golgi complex have been functionally and structurally defined: the cis Golgi network (or intermediate compartment), where vesicles from the ER fuse; the cis Golgi; the medial Golgi where further oligosaccharide processing occurs; the trans Golgi; the trans Golgi network, where proteins are sorted to the lysosome or plasma membrane. These various compartments have been defined on the basis of localization of proteins involved in the modification of the core oligosaccharide added to glycoproteins in the ER (Mellman and Simons, 1992; Alberts *et al.*, 1994) In many mammalian cell types these distinct biochemically defined compartments can be observed in electron micrographs as a set of flattened tubes with dilated rims, and usually arranged in a stack. In yeast the Golgi is also organized into functionally distinct compartments (Franzsuoff and Sheckman, 1989; Graham and Emr, 1991). In general these compartments have been defined either by analysing oligosaccharide modifications of glycoproteins or proteolytic processing of proteins. The isolation of *sec* mutants allowed blocks at various stages of the secretory pathway to be set up as proteins passed through. By studying post-translational modifications the order of processing events could be demonstrated. **Figure 1-2** shows a schematic diagram of the yeast Golgi indicating the presence of various enzymes in each compartment and the modifications made to the proteins pro-α-factor, and carboxypeptidase Y (CPY, a vacuolar hydrolase).

Morphologically the yeast *S. cerevisiae* does not usually have a Golgi stack as seen in mammalian cells although similar stacks are seen in the yeast *S. pombe* (Chappell and Warren, 1989). Yeast Golgi proteins have been localized by immunoelectron

Figure 1-2 A schematic diagram of the compartmental organization of the yeast Golgi. Enzymes which mark three functionally distinct subcompartments are listed to the left of each cisternae (mann= mannosyltransferase). The processing of CPY and pro-α-factor is indicated as they proceed through the pathway. CPY is synthesized as a proenzyme and is core glycosylated in the ER (p1 form). The core oligosaccharide is extended by the mannosyltransferases to produce the p2 form. The p2CPY is sorted from the secretory pathway in the TGN and directed to the vacuole where is is proteolytically matured. Pro-α-factor is also glycosylated as it proceeds through the pathway. In the TGN it is proteolytically processed by Kex2p, Kex1p, and DPAP A. It proceeds along the default pathway for soluble proteins, and is secreted. (adapted from Wilsbach K, and Payne G (1993b).

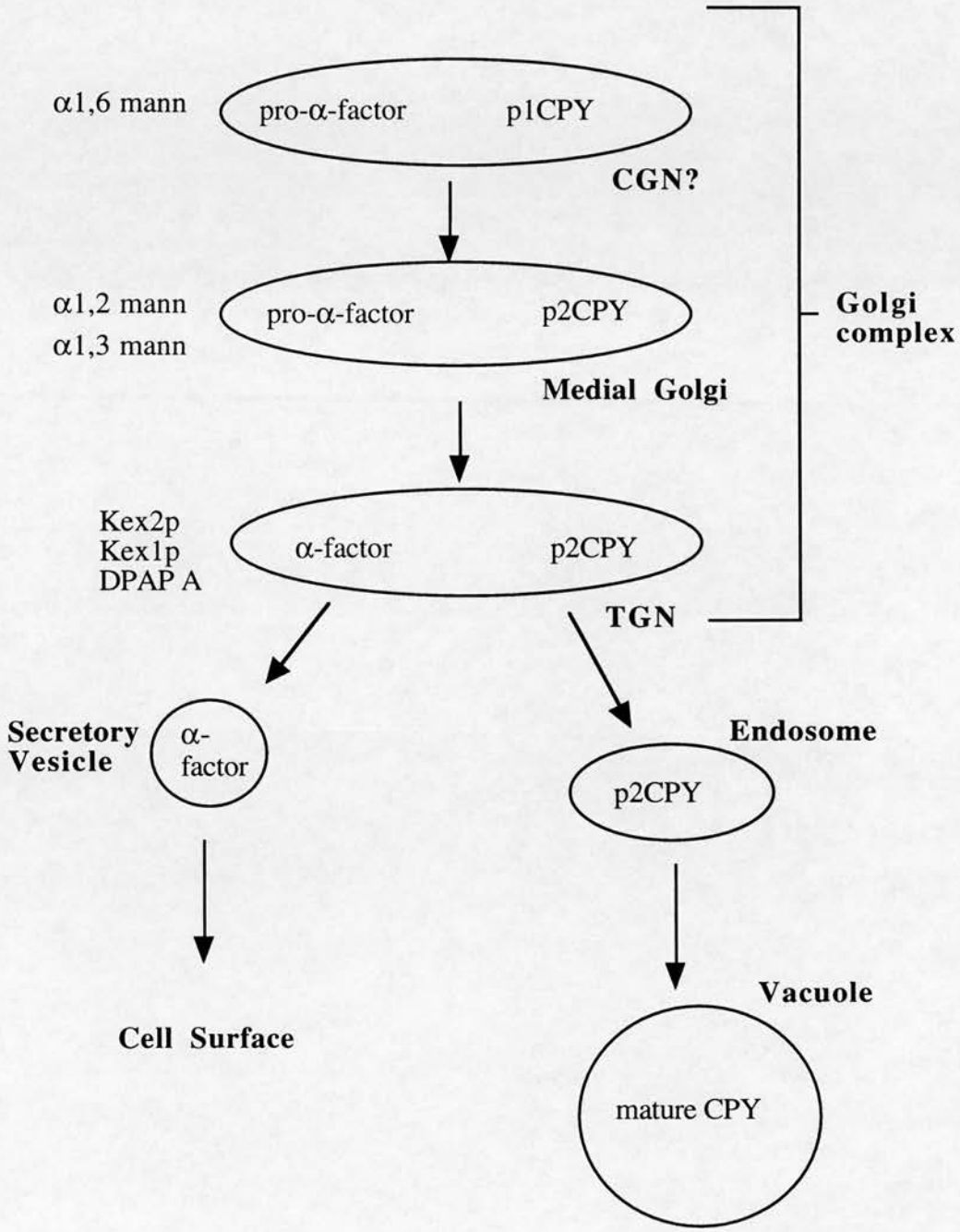


Figure 1-2 Schematic diagram of the compartmental organization of the yeast Golgi.

microscopy to disc-like membranes dispersed throughout the cytoplasm (Preuss *et al.*, 1992) with only occasional clusters of stacks seen. This supports evidence generated by indirect immunofluorescence of Golgi proteins including Kex2p, Ste13p, and Kex1p, (Redding *et al.*, 1991; Roberts *et al.*, 1992; Cooper and Bussey, 1992; Franzusoff *et al.*, 1991; Cleaves *et al.*,1991).

3-2 Retention and retrieval of Golgi proteins

Study of mammalian Golgi glycosylation enzymes has revealed that the transmembrane domain and part of the flanking regions are important for retention (Machamer, 1993). Two models have been described to explain how the transmembrane domain could mediate retention: 1) kin recognition, where proteins of the same compartment associate to form an oligomeric structure which prevents further passage along the secretory pathway (Nilsson *et al.*, 1993; Nilsson and Warren, 1994); 2) lipid composition of compartment membranes. The percentage of cholesterol in a lipid bilayer affects the membrane thickness and variation in the length of the membrane-spanning domain could exclude proteins from certain membranes (Bretscher and Munro, 1993).

Overexpression of various Golgi enzymes does not saturate the retention system and instead of appearing at the cell surface Golgi proteins back up into the ER. The formation of oligomeric structures can explain this observation. Golgi enzymes are proposed to form oligomeric structures via interactions between cytoplasmic domains. The resulting homodimers can interact with each other via their transmembrane domain and luminal domains. This retention mechanism would not be saturated because increased levels of protein would just increase the size of the oligomeric structure. There is growing evidence to support this model. The transmembrane domain from a viral *cis* Golgi protein has been shown to be required for Golgi targeting. When this

domain was used to replace the transmembrane domain from a viral plasma membrane protein Golgi localization was conferred (Machamer *et al.*, 1993). Mutagenesis of the individual amino acids of the transmembrane domain was used to analyze its importance for retention. Four uncharged polar residues were found to be critical for Golgi retention and these residues line one face of a predicted α-helix. It is proposed this face interacts with another identical or similar face, resulting in kin recognition and oligomerization.

There is evidence for a protein matrix between Golgi stacks to which Golgi membrane proteins bind and it could presumably mediate protein retention. The Golgi resists unstacking during purification suggesting a link between adjacent cisternae. The cytoplasmic tails of resident Golgi proteins are not long enough to correspond to such links and so additional proteins must be involved. Slusarewicz *et al.* (1994) have isolated this intercisternal matrix and found it contains 12 major proteins. The matrix binds two medial-Golgi enzymes specifically, N-acetylglucosaminyl transferase I (NAGT I) and mannosidase II. Marker enzymes from other parts of the Golgi are found only in low amounts bound to this matrix. NAGT I has been mislocalized to the ER by addition of the luminal domain of an ER resident protein (Nilsson *et al.*, 1994) which results in another medial enzyme, mannosidase II, also accumulating in the ER. This strongly suggests that kin recognition is involved in retention of these two enzymes. Importantly when a TGN protein, β-1,4-galactosyltransferase was retained in the ER there was no retention of medial enzymes. Furthermore, as levels of the ER form of NAGT I were increased recognizable Golgi stacks disappeared. This can be explained if the medial Golgi enzymes are involved in interacting with the intercisternal matrix. Depletion of the enzymes as they accumulate in the ER would lead to breakdown of Golgi structure. Kin recognition may also play a role in yeast Golgi and ER retention although this remains to be demonstrated. An intercisternal matrix would

however be unlikely to be involved because of the morphology of *S. cerevisiae* Golgi which does not form stacks. *S. pombe* however may have such a matrix, explaining the stacked Golgi in this yeast. Identification of the proteins involved in the mammalian matrix should allow the presence of yeast homologues to be determined.

The primary sequence of transmembrane domains is not important for Golgi localization. The entire transmembrane domain of α2,6-sialyltransferase can be replaced with a polyleucine sequence and Golgi retention is unaffected (Munro, 1991; Dahdal and Colley, 1993). What does seem to be important is the length of the transmembrane domain (Masibay *et al.*, 1993). In general the length of the transmembrane domain is shorter in Golgi proteins than those found in plasma membrane proteins. This has led to the idea that thickness of a lipid bilayer is in part responsible for membrane protein retention. Orci *et al.* (1981) demonstrated that a gradient of cholesterol exists through the secretory pathway and the more cholesterol a bilayer contains the thicker the membrane. The significance of this gradient may be to allow membranes to have different permeabilities. The ER membrane has a low cholesterol content resulting in a more permeable membrane, which is presumably desirable to allow translocation of proteins. The plasma membrane however needs to be less permeable to maintain cellular integrity and it contains a much higher cholesterol level (Bretscher and Munro, 1993). Bretscher and Munro suggest that Golgi membrane proteins could be excluded from vesicles intended for later compartments on the basis of length of transmembrane domain. Early *cis* Golgi proteins would have a short transmembrane domain which would make it energetically unfavourable for them to insert into membranes which contained more cholesterol and were therefore thicker. It would seem likely that a combination of membrane thickness and kin recognition is responsible for Golgi protein retention.

3-3 Retention and protein sorting in the TGN

The TGN is a late Golgi compartment where in mammalian cells proteins are sorted to the lysosome or plasma membrane, and in yeast to the vacuole or plasma membrane. Studies on the mammalian TGN protein TGN38 have found a localization signal in its cytoplasmic tail (Bos *et al.*, 1993; Humphrey *et al.*, 1993; Wong and Hong, 1993). Deletion or point mutation of this tyrosine based signal leads to TGN38 appearance at the plasma membrane.

Work in yeast has indicated that the mechanisms of protein sorting and retention in the TGN are closely linked (Wilsbach and Payne, 1993). Mutagenesis of the cytoplasmic tails of various TGN proteins has identified retention signals. The Kex2p retention signal consists of an essential tyrosine and flanking sequence similar to that found in TGN38 (Wilcox *et al.*, 1992). DPAP A (or Ste13p) contains a signal based around the sequence FQFND (Roberts *et al.*, 1992; Nothwehr *et al.*, 1993). Surprisingly when these retention signals were mutated the mutant proteins were transported to the vacuole rather than the plasma membrane. In mammalian cells the default pathway is to the plasma membrane with transport to the lysosome requiring a specific sorting signal. In yeast soluble vacuolar proteins are known to require a sorting signal, the default for soluble proteins being secretion from the cell (Valls *et al.*, 1990). When the vacuolar membrane protein DPAP B was examined for vacuolar targeting signals no part of the protein could be identified which was required for vacuolar delivery (Roberts *et al.*, 1992). Together the evidence outlined above has supported the model that in yeast the default pathway for membrane proteins is to the vacuole and not to the plasma membrane as is the case in mammalian cells. This model predicts that yeast plasma membrane proteins will contain sorting/targeting signals. So far none have been reported. The DPAP A Golgi retention signal is able to localize the vacuolar membrane protein alkaline phosphatase (ALP) to the TGN. How this signal and the

Kex2p signal mediate retention is unclear but competition experiments suggest a common feature is involved, (Nothwehr *et al.*, 1993): when Kex2p is overexpressed the DPAP A-ALP fusion protein is not as efficiently retained in the TGN suggesting the two proteins are competing for some factor. However if Kex2p retention deficient mutant proteins are overexpressed then the effect on DPAP A-ALP is greatly reduced presumably because the mutant retention signals can no longer efficiently compete with the fusion protein.

3-4 Clathrin and TGN protein retention

Clathrin-coated vesicles mediate receptor-mediated endocytosis and sorting of soluble proteins from the TGN to the lysosome (in mammalian cells) or vacuole (in yeast). Clathrin consists of three heavy chain and three light chain subunits (Pearse and Robinson, 1990) which are conserved throughout eukaryotes. When the yeast clathrin heavy chain subunit gene (*CHC1*) was deleted a mating defect was observed (Payne and Sheckman, 1989). α-strains secrete a mating pheromone α-factor which is synthesized as a precursor and processed in the Golgi (Sprague and Thorner, 1993). The *chc1* strain was found to secrete α-factor as the highly glycosylated precursor form, similar to that secreted by a *kex2* mutant, suggesting that the precursor had not encountered the protease Kex2p in the TGN (the *KEX2* gene encodes an endoprotease involved in α-factor processing (Sprague and Thorner, 1993)). When the localization of Kex2p was examined in a *chc1* mutant it was found at the cell surface rather than in the TGN. Payne and Sheckman (1989) proposed that clathrin was involved in Kex2p retention in the TGN probably through interaction with the cytoplasmic tail of Kex2p and that this retention could be either a stable tethering event or a recycling event. Seeger and Payne (1992) examined the effects on other Golgi and vacuolar proteins in *chc1* mutants and temperature-sensitive *chc1* mutants. They showed that about 60-90% of Kex2p mislocalized to the cell surface. 30% of another

endoprotease (DPAP A) involved in TGN α-factor processing also mislocalized to the plasma membrane. In contrast GDPase, a type II membrane protein found in an early Golgi compartment (Aberijon *et al.*, 1989) was not mislocalized. This suggests that clathrin is involved in retention of a subset of Golgi proteins.

In clathrin mutants TGN proteins are found at the cell surface. How can this be explained in light of the evidence for a vacuolar default pathway for membrane proteins? One explanation would be if clathrin mutants have a defect in sorting of proteins to the vacuole thereby blocking the default pathway. Seeger and Payne (1992b) examined sorting to the vacuole in a *chc1-ts* strain at the restrictive temperature (37^{o}C) and found that soluble protein sorting was defective but that vacuolar membrane proteins were delivered normally. These results fail to explain why clathrin mutants deliver TGN proteins to the plasma membrane and not to the vacuole. Interestingly after extended growth at 37^{o}C *chc1-ts* regained the ability to sort the soluble vacuolar protein CPY normally to the vacuole. This observation and the normal delivery of vacuolar membrane proteins suggests the presence of a clathrin-independent sorting mechanism. This could explain why *chc1Δ* mutants do not have soluble vacuolar protein sorting defect.

The mammalian protein TGN38 is known to recycle between the TGN and plasma membrane (Bos *et al.*, 1993). If TGN proteins follow a similar pathway in yeast then loss of clathrin might be expected to result in plasma membrane localization. However there is no evidence for yeast TGN proteins at the plasma membrane nor do they accumulate in vesicles accumulated in a *sec1* block, which blocks TGN to plasma membrane transport (Redding *et al.*, 1991). The appearance of TGN proteins at the plasma membrane in *chc1* mutants does not contradict the vacuolar default pathway model discussed earlier. If clathrin is not present vacuolar bound vesicles cannot form

and therefore TGN membrane proteins follow the secretory pathway to the plasma membrane when retention is interfered with.

The role of clathrin in protein retention is far from clear as recent work demonstrates. The α1,3 Mannosyltransferase (Mnn1p) has been localized (Graham *et al.*, 1994) to the Golgi in yeast, and sucrose gradient fractionation reveals two physically distinct compartments. One colocalizes with early Golgi markers and the other with TGN markers. When the localization was examined in a *chc1*-ts strain, at the restrictive temperature, 40-80% of Mnn1p was found at the cell surface. Graham *et al.* (1994) predicted that the cytoplasmic tail of Mnn1p would contain a retention motif similar to those described in Kex2p and DPAP A and that this motif would be required for efficient retention probably through a direct interaction with clathrin. However the cytoplasmic tail of Mnn1p contained no obvious retention motif and resembled the short tail of GDPase rather than the longer tails of Kex2p and DPAP A. When the cytoplasmic tail of Mnn1p was replaced using a tail from a plasma membrane protein the fusion protein maintained a Golgi localization instead of the plasma membrane or vacuolar localization predicted if the tail was important in retention. Graham *et al.* (1994) propose three models to explain this surprising result: 1) the entire Kex2p containing compartment is lost to the plasma membrane in a *chc1* mutant and any Mnn1p found here is localized to the plasma membrane (No direct evidence for colocalization of Kex2p with Mnn1p is presented); 2) Mnn1p interacts with another protein(s) which directly interacts with clathrin. Loss of clathrin and therefore this protein(s) would indirectly result in Mnn1p expression at the plasma membrane; 3) loss of clathrin affects retention of proteins required to maintain the lipid characteristics of Golgi stack membranes. This could alter cholesterol content, for example, which may be critical for Golgi protein retention (see section **3-2**).

3-5 *VPS* gene products important in TGN function

Various genetic selection schemes have isolated a large number of mutants defective in vacuolar protein sorting (Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989). These vacuolar protein sorting defective mutants (*vps* mutants) missort and secrete soluble precursor vacuolar hydrolases into the growth medium. More than 40 complementation groups have been identified (Klionsky *et al.*, 1990). The various alleles have been characterized according to the vacuolar morphology they induce (Raymond *et al.*, 1992). The localization of the proteins encoded by *VPS* genes has also been characterized. This section outlines some recent findings on the role of several *VPS* gene products in the secretory pathway.

3-6 *VPS1*: importance in retention of TGN proteins and vacuolar protein sorting

As previously discussed Kex2p is mislocalized to the plasma membrane in *chc1* mutants. The result is secretion of unprocessed α-factor into the growth medium. A screen for mutants defective in Kex2p retention was based on this observation and this screen identified the *VPS1* gene product as essential for Kex2p retention. In contrast to *chc1* mutants, *vps1* mutants do not mislocalize Kex2p to the plasma membrane but to the vacuole instead (Wilsbach and Payne, 1993a). It appears that both Vps1p and Chc1p are involved in Kex2p retention but how are the different mislocalization phenotypes explained The discovery of a prevacuolar compartment has suggested a model to answer this question and is discussed below.

vps mutants have been classified according to vacuolar morphology (Raymond *et al.*, 1992) and the class E *vps* mutants accumulate a distinctive membrane organelle next to the vacuole. Internalized α-factor accumulates in this compartment (Davis *et al.*, 1993) and subcellular fractionation suggests the compartment exists in wild type cells

(Vida *et al.*, 1993). This evidence has led to the proposal of a prevacuolar compartment where the endocytic pathway and vacuolar protein sorting pathway converge. It has been suggested that if TGN proteins are cycling between the TGN and prevacuolar compartment (in a manner similar to that of ER proteins which cycle between the ER and intermediate compartment) the different fates of Kex2p in *vps1* and *chc1* mutants can be explained (Wilsbach and Payne, 1993a; Wilsbach and Payne, 1993b). The model proposes that TGN membrane proteins and soluble vacuolar precursor proteins are sorted into clathrin-coated vesicles destined for the vacuole. This sorting is mediated by interactions between the cytoplasmic tails of TGN proteins (Kex2p and DPAP A) and by receptors for the soluble proteins interacting with the clathrin coat (see section on *VPS10*). The coated vesicles deliver their cargo to the prevacuolar compartment where the soluble vacuolar precursors dissociate from their receptors and are transferred to the vacuole. The receptors and the TGN membrane proteins however are recycled back to the TGN ready for another round of transport and this recycling back to the TGN is proposed to be Vps1p dependent. Vps1p is a member of a family of high molecular weight GTPases which includes the dynamin protein and a *Drosophila* homologue, the *shibire* protein. Both these proteins play a role in the formation of endocytic vesicles and Vps1p might therefore act in vacuolar protein sorting and Golgi protein retention through a role in biogenesis of transport vesicles.

So how exactly does the model outlined account for the Kex2p localization in *vps1* and *chc1* mutants? If clathrin is mutated, sorting of TGN proteins and vacuolar precursors from the TGN to the prevacuolar compartment is prevented. This results in packaging into secretory vesicles and hence the observed cell surface expression of Kex2p and *vps* phenotype observed. In a *vps1* mutant TGN proteins and the receptors for soluble vacuolar precursor proteins are sorted correctly to the prevacuolar compartment.

However on delivery to the prevacuolar compartment the TGN proteins and receptor molecules cannot be returned to the TGN because this is Vps1p dependent and the result is delivery of Kex2p to the vacuole.

One major fault with the model described above is that it cannot explain the vacuolar default pathway for membrane proteins. TGN proteins with mutations in their cytoplasmic tails should not be able to interact with clathrin and should therefore not enter the vacuolar sorting pathway. However it is clear that Kex2p, DPAP A, and Kex1p all mislocalize to the vacuole when their cytoplasmic tails are mutated or deleted. One possibility is that a clathrin-independent mechanism is responsible for the sorting of mutant membrane proteins. The sorting of the vacuolar membrane protein alkaline phosphatase (ALP) is not affected in *chc1* mutants (Seeger and Payne, 1992a, 1992b), and furthermore *chc1-ts* mutants have been shown to adapt to loss of clathrin and soon lose their *vps* phenotype. It is clear the model outlined above is incomplete and cannot account fully for all the observations made so far.

3-7 *VPS10* encodes a putative CPY receptor

As mentioned in the previous section soluble vacuolar precursor proteins are thought to be sorted to the vacuole by receptor molecules in the TGN which interact with clathrin. In the case of CPY a targeting signal has been defined with a sequence QRPL (Valls *et al.*, 1987). The presence of a receptor mediated system is supported by the observations that the sorting of CPY is saturable, and specific for CPY (Stevens *et al.*, 1986; Rothman *et al.*, 1986). In order to identify a possible CPY receptor a screen of existing *vps* mutants which specifically missort CPY and not multiple vacuolar hydrolases (PrA, PrB, and ALP) was carried out. This screen identified *vps10*, *vps 29*, and *vps35* as possible candidates (Paravincini *et al.*, 1992). Recently the *VPS10* gene has been shown to encode the CPY sorting receptor (Marcusson *et al.*,

1994). Vps10p is predicted to be a type I transmembrane protein and cofractionates with Kex2p, a TGN protein. Marcusson *et al.* (1994) demonstrated that Vps10p specifically binds the Golgi modified p2 form of CPY by interaction with the QRPL sequence.

4 Vesicular transport

Vesicular transport of proteins between compartments, such as the ER and Golgi, must be a highly specific and targeted process. Studies on neuronal exocytosis, biochemical analysis of *in vitro* transport studies, and yeast genetics have all identified components performing steps in this process (Rothman and Orci, 1992; Pryer *et al.*,1992; Ferro-Novick and Jahn, 1994). Insights into vesicle budding, targeting and fusion have been gained and some recent and illustrative advances are discussed below.

4-1 Vesicle budding

Most transport vesicles are thought to form when proteins called coat proteins are recruited to specific areas of membranes. These coated regions bud off from the membrane to form coated vesicles. There are two well characterized types of coated vesicles, clathrin-coated vesicles, and coatomer-coated vesicles. Clathrin-coated vesicles mediate transport between the plasma membrane, endocytic, and trans-Golgi compartments (Pearse and Robinson, 1990; Anderson, 1992). The forces generated by the self assembly of the clathrin coat are thought to drive bud formation. Other major coat proteins found in these vesicles are the adaptors. Adaptor complexes are thought to trap various transmembrane proteins in the coated membrane before budding (Robinson, 1994). If these proteins are receptors for specific luminal cargo molecules then this ensures they are packaged into the vesicle. In general clathrin-coated vesicles are responsible for selective vesicular transport such as occurs at the

TGN where proteins are sorted.

In contrast coatomer-coated vesicles mediate nonselective vesicular transport such as that from the ER to Golgi and between the Golgi stacks. The coat of these vesicles is made of proteins called COPs. COPs do not self-assemble like clathrin but require energy. Assembly of coatomer coats is thought to require the activity of various GTP-binding proteins which are in part responsible for the specificity and unidirectional nature of vesicle transport. COP I coated vesicles are responsible for intra-Golgi coated vesicle transport (Waters *et al.*, 1991; Pryer *et al.*, 1992) however to illustrate the processes involved in vesicle budding, the budding of vesicles from the ER membrane of yeast will be discussed in some detail. These recent experiments have identified a new class of coated vesicle, COP II coated vesicles, and indicate that processes involved in formation of all coated vesicles are conserved. This work also serves to illustrate the power of yeast genetics coupled with biochemical analysis of cell-free systems.

The original screen for *sec* mutants (Novick *et al.*, 1980,1981) revealed several genes which were required for vesicle budding from ER membranes. Other more recent screens have also revealed genes required for vesicle budding (Pryer *et al.*, 1992). Functional *SEC12*, *13*, *16*, *23*, and *SAR1* gene products are all required for vesicle formation from the ER in yeast. An *in vitro* budding reaction using gently lysed yeast spheroplasts has been developed (Rexach and Schekman, 1991). In this reaction an *in vitro* translated radiolabelled secretory protein precursor is translocated into the ER. This protein is pre-pro-α-factor, the precursor for the mating pheromone α–factor. By using this assay the requirements for budding, targeting, and fusion of ER-derived transport vesicles were investigated by adding back various cytosolic components and following the subsequent movement of pre-pro-α-factor through the pathway as

revealed by post-translational modifications to the protein.

In this way it has been shown that three cytosolic fractions are required for vesicle budding from the ER: Sar1p; a complex of Sec23p/Sec24p; and a complex of Sec13p/p150 (Salama *et al.*, 1993). Sar1p is a 21 kDa GTPase (Nakano and Muramatsu, 1989) which is activated by a GDP/GTP exchange on the cytosolic face of the ER catalysed by an integral membrane protein Sec12p (Barlowe and Sheckman, 1993). Sec23p is a Sar1p-specific GTPase-activating protein (GAP), and Sec24p has an unknown function (Yoshihisa *et al.*,1993). These three components of the coatomer have been termed COP II.

Examination of these COP II-coated vesicles by electron microscopy reveals vesicles of about 60 nm diameter with a homogeneous amorphous coat (Barlowe *et al.*, 1994). A mechanism for ER vesicle budding has been proposed based on recent experiments by several groups (Barlowe *et al.*, 1994; Oka and Nakamo, 1994). Sar1p is recruited onto the ER membrane where Sec12p catalyses the exchange of GDP for GTP. The *SAR1* gene is a high copy suppressor of *sec12* and if Sar1p is incubated with a non-hydrolysable analogue of GTP (GTPγS) it is unable to suppress a *sec12* mutation in an *in vitro* transport assay (Oka and Nakoma, 1994). Interestingly this proved to be a defect in vesicle targeting rather than vesicle budding. The presence of Sar1p on the ER membrane drives the recruitment of the Sec13p/p150, and Sec23p/24p complexes. In terms of molecular sizes these complexes resemble clathrin and adaptors respectively although no protein sequence homology exists. The recruitment of these two complexes in some way drives vesicle budding. After budding Sec23p, which has a GAP activity hydrolyses, the Sar1p-bound GTP causing Sar1p to dissociate and this step is required to produce fusion competent vesicles.

COP I-mediated vesicle budding (in mammalian cells) from Golgi membranes occurs in a very similar manner (Ostermann *et al.*, 1993). Again the first step is the binding of a small GTP-binding protein ADP-Ribosylation Factor 1 (ARF1) to the membrane. ARF1 recruitment requires GTP to be bound and this is performed by a protein equivalent to Sec12p. After ARF1 binding to the membrane the coatomer complex forms from the various COPs and A RF1 and the COPs are referred to as COP I. ARF1 is also required for recruitment of clathrin and adaptors at the TGN (Stamnes and Rothman, 1993) and it would seem that vesicle budding has a universal requirement for small GTP-binding proteins. The yeast equivalent of COP I has been identified and the *SEC21* gene encodes the γ-COP component (Hosobuch *et al.*, 1992). Depletion of coatomer from the membranes involved in the *in vitro* budding assay did not affect formation of the ER-derived transport vesicles, however *sec21* mutants are blocked in ER to Golgi transport. This together with observations in mammalian cells that antibodies to β-COP, and ARF1 mutants are also blocked at a pre-Golgi step (Pepperkok *et al.*, 1993; Peter *et al.*, 1993) suggests COP I is also involved in ER to Golgi transport. Several explanations are offered by Barlowe *et al.* (1994) for this apparent discrepancy: their *in vitro* system does not accurately reflect those *in vivo*; COP I and COP II both act at the ER but package different cargoes; COP I is involved in the pathway from the Golgi back to the ER. Another possibility is that COP II is involved in vesicle budding from the ER and transport to an intermediate compartment and COP I forms coated vesicles from here. There is immunolocalization evidence to support this proposal (discussed by Seaman and Robinson, 1994).

Several other observations were made from the *in vitro* budding assay (Barlowe *et al.*, 1994). When the contents of the purified COP II coated vesicles were examined they were found to be enriched for certain proteins and lacking others. For example Sec61p, Kar2p (BiP), and Sec12p are all absent from COP II vesicles. On the other

hand several membrane proteins required for vesicle targeting (discussed in section **5-2**) are detected, in particular Bos1p, Sec22p, and Ypt1p. The contents of a COP II vesicle may be determined simply by the ER retention, retrieval system outlined earlier. An alternative is that COP II-coated vesicles may contain adaptor like molecules which specifically package certain ER proteins.

5 Vesicular targeting

5-1 NSF and SNAPs

Mammalian cell-free transport assays identified a requirement for an N-ethlymaleimide (NEM) sensitive component in membrane fusion. This NEM-sensitive fusion (NSF) protein which was purified on the basis of its ability to restore transport in assays using NEM-pretreated Golgi membranes (Block *et al.*, 1988), and was shown to be very similar to the product of the *SEC18* gene in yeast (Sec18p has now been shown to have NSF activity ; Wilson *et al.*, 1989). NSF is involved at multiple steps in the secretory pathway including between the ER and Golgi, the trans-Golgi and TGN, and at some stage during receptor-mediated endocytosis (Beckers *et al.*, 1989; Diaz *et al.*, 1989). NSF could be purified in a membrane-bound form, but its amino acid sequence predicted a water soluble molecule, and it could indeed be purified in this form as well (Wilson *et al.*, 1989; Block *et al.*,1988). This suggested that NSF might cycle between a membrane-bound and a soluble form, binding membranes to promote fusion. It was demonstrated that pure NSF could not bind Golgi membranes (Weidman *et al.*, 1989) but that addition of crude cytosol enabled NSF to bind. A family of proteins have now been described which are required to bind to membranes before NSF is recruited (Clary *et al.*, 1990). These soluble NSF attachment proteins (SNAPs) are closely related peripheral membrane proteins and bind to specific SNAP receptors (SNAREs). The *SEC17* gene in yeast has been identified as encoding the functional homologue of α-SNAP from mammalian cells (Clary *et al.*, 1990). The

fact that NSF and SNAPs seem to act at multiple stages of the secretory pathway suggested that other molecules were responsible for producing the specificity in targeting of vesicles. Attention has concentrated on the small GTP-binding proteins (Rabs) and more recently on the SNAREs.

5-2 The SNARE hypothesis

Purification of a set of proteins termed SNAREs has shed light on how vesicle targeting may be performed. A complex of recombinant NSF and SNAP proteins was used to isolate SNAREs from detergent-solubilized brain cell membranes (Sollner *et al.*, 1993). Briefly, NSF was tagged with the c-*myc* epitope and bound via anti-myc IgG to a solid bead matrix. This matrix was incubated with detergent solubilized membranes and SNAPs and after various washes and elution procedures three SNARE molecules were isolated. All three proteins had been previously identified as components of synaptic membranes. Vesicle-associated membrane protein (VAMP) or synaptobrevin is found only in synaptic vesicles (Trimble *et al.*, 1988; Baumert *et al.*, 1989). Syntaxin and SNAP-25 were the other two proteins and they are found in the pre-synaptic plasma membrane (Bennet *et al.*, 1992; Oyler *et al.*,1989). The exclusive distributions of these proteins suggested the SNARE hypothesis. In this model transport vesicles contain a unique vesicle-SNARE (v-SNARE) which matches a precise target-SNARE (t-SNARE) in the intended target membrane (Rothman and Warren, 1994). It is predicted that each step in the secretory pathway has its own v-SNARE and t-SNARE and that the v-SNAREs will be VAMP- like molecules, and the t-SNAREs syntaxin/ SNAP-25-like molecules.

The SNARE hypothesis is strongly supported by recent biochemical and genetic evidence. A protein complex containing stoichometric amounts of VAMP, syntaxin, and SNAP-25 is formed in detergent-solublized brain cell extracts (Sollner *et al.*,

1993). The ATPase activity of NSF disrupts this complex which may be of importance *in vivo* because at some stage the stable SNARE complex must presumably be broken up to allow membrane fusion to occur. Other evidence for the SNARE hypothesis is that various neurotoxins which affect exocytosis *e.g.* botulinum A and E have been shown to selectively cleave synaptic SNAREs (Schiavo *et al.*, 1992; Blasi *et al.*, 1993a, 1993b). Further evidence comes from the identification of SNARE homologues in non-neuronal cells including yeast. In mammalian cells, homologues of VAMP, syntaxin, and SNAP-25 have all been described (McMahon *et al.*, 1993; Bennet *et al.*, 1993; De Camilli 1993). In yeast COP II-coated vesicles derived from the ER have been shown to contain the *BOS1* gene product (Barlowe *et al.*, 1994), Bos1p which is a homologue of the v-SNARE, VAMP (Lian and Ferro-Novick, 1993). *SED5* mutants also accumulate ER-derived vesicles and Sed5p is a homologue of the t-SNARE, syntaxin and is found in the *cis*-Golgi (Hardwick and Pelham, 1992). An interaction between Sed5p and Bos1p has yet to be demonstrated. SNAREs have also been identified which are involved in Golgi to plasma membrane transport (Protopopov *et al.*, 1993; Aalto *et al.*, 1993). The *SNC1/SNC2* gene products are VAMP homologues (v-SNAREs) and the *SSO1/SSO2* gene products syntaxin homologues (t-SNAREs).

6 The importance of Rabs

Small GTP-binding proteins of the Rab family are of critical importance to vesicular transport, although emphasis on their role has shifted (Pryer *et al.*, 1992; Zerial and Stenmark, 1993 ; Pfeffer, 1994). Sec4p and Ypt1p were the first Rabs described and were identified in yeast. They are 48% identical but temperature-sensitive mutants are blocked at different points in the secretory pathway at the restrictive temperature. *ypt1* mutants are blocked in ER to Golgi transport whereas *sec4* mutants are blocked in Golgi to plasma membrane transport. Sec4p has been localized to both the plasma

membrane and the cytosolic face of secretory membranes (Goud *et al.*, 1988). Many mammalian Rab genes have since been isolated on the basis of homology to *ras* genes and to *SEC4* and *YPT1*. Localization of Rab proteins showed they had a highly restricted distribution and led to the hypothesis that different GTP-binding proteins act at each step in the secretory pathway.

Cell-free assays have been used to demonstrate the requirement for Rab proteins. Non-hydrolyzable GTP analogues, anti-Rab antibodies, and Rab peptide mimics all blocked mammalian *in vitro* assays (Pryer *et al.*, 1992). It is proposed that Rab proteins are involved in ensuring the directionality of the vesicle docking/fusion process and that the cycle of GTP binding and hydrolysis is responsible for this (Bourne, 1988; Pfeffer, 1992). Rab proteins are thought to be recruited onto membranes in a GTP-bound form, with a geranylgeranyl motif at their C-terminus mediating the membrane association. Hydrolysis of GTP by the Rab is then thought to trigger fusion of transport vesicles with the target membranes. The Rab-GDP form is then released for another cycle of transport. The hydrolysis of GTP is performed by the Rab but requires the activity of a GTPase-activating protein (GAP). Another protein, guanine-nucleotide dissociation inhibitor (GDI) is thought to retrieve the GDP-bound Rab protein from membranes and escort them back to the donor membrane.

Although Rabs are clearly important in vesicular transport a model where they act as unique identifiers is unlikely. A more likely model is that they are part of a complex of proteins required for proper targeting (Bourne, 1988). Rabs are often localized uniquely but there is an increasing number of examples of Rabs localized to multiple compartments, of multiple Rabs localized to the same compartment, and of the same Rab being required at several stages of transport (Rothman and Warren, 1994). In

yeast a hybrid Rab has been constructed from Ypt1p and Sec4p which is able to substitute for wild type Ypt1 and Sec4p without affecting normal vesicular transport (Brennwald and Novick, 1993; Dunn *et al.*, 1993). It is now proposed that SNAREs mediate the specificity of targeting but that Rabs are involved in an extra proof-reading process. Two recent papers shed light on the SNARE hypothesis with particular reference to the involvement of Rabs in yeast vesicular transport and are discussed briefly below.

The components of a SNARE complex(es) have been investigated by accumulating ER-derived vesicles in a *sec18* mutant (Sogaard *et al.*, 1994). The *SEC18* gene encodes the yeast homologue of NSF (see section **5-1**) and Sec18p is required at multiple stages of the secretory pathway. When *sec18* mutants are incubated at the restrictive temperature, fusion of docked vesicles with the Golgi membrane is blocked. When antibodies against the proposed Golgi t-SNARE Sed5p were used to immunoprecipitate from whole-cell extracts prepared from *sec18* mutants (grown at the restrictive temperature) a total of nine specific polypeptides were observed (Sogaard *et al.*, 1994). These proteins were identified by micro-sequencing and Western blotting with affinity-purified antibodies as: Sed5p; Sly1p (a Sec1p homologue); Sec17p (a SNAP homologue); Bos1p, Sec22p and Bet1p (all proposed v-SNAREs); and three previously undescribed proteins p28, p26, and p14. p26 was a VAMP homologue (v-SNARE) previously identified as Ykt6p. Ykt6p is a predicted peripheral membrane protein with a potential farnesyaltion site and is the first SNARE described not to have a transmembrane sequence. Sogaard *et al.* (1994) went on to demonstrate that this complex(es) did not accumulate when a v-SNARE was inactivated by showing that *sec22* mutants did not form a complex when incubated at 37^{o}C. They also showed that Sec17p was required for either the stability or formation of the complex(es). Ypt1p is a Rab protein required for ER to Golgi transport (Segev

et al., 1988) and is required for attachment and/or fusion of ER- derived transport vesicles (Segev, 1991; Rexach and Sheckman, 1991). Sogaard *et al.* showed that the SNARE complex(es) was unable to form in *ypt1* mutants at the restrictive temperature and since all the major suppressors of *ypt1* (the *SLY* genes) bind to either t- or v-SNAREs they suggest that Ypt1p most likely acts directly on the SNARE complex to allow assembly. Ypt1p was not found in the SNARE complex so appears to have a role in assembly only. The presence of so many SNAREs in a single type of vesicle raises some interesting questions. It could be that to ensure absolute specificity of targeting multiple SNAREs are required. It also suggests a possible mechanism for targeting of several vesicle types using a limited number of SNAREs, various combinations of the same SNAREs could specify different targets.

A genetic and biochemical interaction between another Rab protein (Sec4p) and a SNARE complex has been recently described. The *SEC4* gene encodes another yeast Rab protein and *sec4* mutants are blocked in Golgi to plasma membrane traffic. Brennwald *et al.* (1994) mutated the effector domain of Sec4p and isolated high-copy suppressors of *sec4*, identifying the *SEC9* gene as the most efficient suppressor. This gene is essential and analysis of its sequence revealed it's C-terminus to be 60% similar to SNAP-25, a mammalian plasma membrane t-SNARE. Sec9p localizes to the plasma membrane and associates with the yeast homologues of syntaxin and synaptobrevin (*SSO1/SSO2*, *SNC1/SNC2* gene products).

7-1 The importance of Ca^{2+} homeostasis

Many cellular processes are governed by changes in Ca^{2+} levels within the cell (Alberts *et al.*, 1994). The concentration of cytosolic free Ca^{2+} can control specialized functions like excitability, contraction, and exocytosis, as well as regulating universal cellular activities such as metabolism and gene expression. The

release of Ca^{2+} from intracellular stores in response to inositol 1,4,5-trisphosphate (IP3) is perhaps the best characterized event (Taylort and Marshall, 1992; Michell, 1992). IP3 links receptor coupled G-proteins to Ca^{2+} release which affects various soluble or membrane-bound effector proteins (eg calmodulin). The cytosolic concentration of Ca^{2+} is maintained at extremely low levels ($< 10^{-7}$ M) allowing even small changes to be detected in the cell. Ca^{2+} is in fact toxic to cells at prolonged high concentrations, and the cytosol is strongly buffered to resist changes in Ca^{2+} concentration. This is achieved by Ca^{2+}ATPases in the plasma membrane which use the energy from ATP hydrolysis to pump Ca^{2+} out of cells (Carafoli, 1987; Tsien and Tsien, 1990; Alberts *et al.*, 1994). Muscle and nerve cells have a Na^{+}-driven Ca^{2+} pump which couples the influx of Na^{+} to Ca^{2+} efflux.

7-2 The importance of Ca^{2+} in the secretory pathway

The organelles of the secretory pathway play a crucial role in the control of cellular Ca^{2+} homeostasis. In mammalian cells the main intracellular stores are the ER or sarcoplasmic reticulum (SR) (Somlyo *et al.*, 1985; Tsien and Tsien, 1990; Alberts *et al.*, 1994). ATP-driven Ca^{2+} pumps in the ER and SR can pump Ca^{2+} into the lumens of these organelles even against a steep concentration gradient. Potential Ca^{2+} pumps have been localized to the Golgi (Virk *et al.*, 1985; Antebi and Fink, 1992), lysosomes/ vacuoles (Klemper, 1985; Cunningham and Fink, 1994), secretory granules (King *et al.*, 1988), and endocytic compartments (Milne and Coukell, 1989). In most cases the physiological function of these pumps has not been determined.

7-3 Ca^{2+} in the ER

In mammalian cells the ER is a major store of Ca^{2+} ions, mainly bound to high-capacity, low affinity proteins such as calreticulin (Koch, 1987; Macer and Koch, 1988). The retention of ER resident proteins is affected by changes in Ca^{2+} levels.

Booth and Koch (1989) showed that Ca^{2+} ionophores cause loss of retention of various ER proteins (*eg* PDI, BiP, and calreticulin) in NIH 3T3 cells. Ionophores specific for other ions had no effect. It seems likely that Ca^{2+} is in some way involved in retention or retrieval of ER resident proteins (see section **2**). What is difficult to explain is that prolonged stress caused by Ca^{2+} ionophores actually results in reduced secretion of ER proteins (Booth and Koch, 1989; Macer and Koch, 1988). It may be that by increasing the levels of proteins in the ER, retention is improved due to increased physical interactions. These interactions may act to prevent packaging into transport vesicles.

Ca^{2+} ionophores have also been shown to block exit of secretory proteins from the ER (Lodish and Kong, 1990). α1-antitrypsin, antichymotrypsin and albumin all have their secretion impaired in human hepatoma (HepG2) cells by ionophores. In agreement with Booth and Koch (1989) increased levels of BiP were detected. BiP binds transiently to newly synthesized wild type proteins as well as more permanently to malfolded or unassembled protein whose transport from the ER is blocked (Gething and Sambrook, 1992). The association of BiP with proteins is dependent on Ca^{2+} levels in the ER. Ca^{2+} ionophores result in the release of stably retained T-cell receptor (TCR) variants (Suzuki *et al.*, 1991). These variants are bound to BiP in the ER, however no BiP is bound to the TCRs nor is BiP secreted on treatment of cells with ionophores. This observation suggests that the increased levels of BiP seen in the HepG2 cells is not responsible for the block in secretion. Lodish and Kong (1990) proposed that depletion of Ca^{2+} resulted in malfolded proteins being retained by BiP. Although this may not be the case there are other resident stress-induced proteins which could perform this function.

Ca^{2+} ionophores have also been shown to cause an increase in protein degradation in

the ER (Wileman *et al.*, 1991). A three-fold increase in the rate of degradation of TCRs and CD3-δ was observed in CHO cells. It is not clear whether this is because control of proteolysis is perturbed by depletion of Ca^{2+}, or whether due to increased levels of malfolded proteins, proteolysis is increased.

7-4 Vesicular transport requires Ca^{2+}

A requirement for Ca^{2+} in many stages of vesicular transport through the secretory pathway has been demonstrated in *in vitro* systems which reconstitute transport between intact organelles. The transport of vesicles from the ER to *cis* Golgi compartment in CHO cells requires GTP and Ca^{2+} (Beckers and Balch, 1989). In semi-intact CHO cells the transport of vesicular stomatitis virus (VSV) G protein between the ER and Golgi was followed by monitoring the processing of the core-oligosaccharide. EGTA was found to prevent Golgi processing of VSV-G protein and this block was fully reversible when Ca^{2+} was added back.

In yeast the GTP-binding protein Ypt1p is required for ER to Golgi traffic (see section **6**) and two studies suggested a link between Ypt1p and Ca^{2+}: 1) high extracellular concentrations of Ca^{2+} suppress the temperature-sensitive growth defect of *ypt1-ts* mutants (Schmitt *et al.*, 1988); and 2) deletion of *PMR1*, a gene which encodes a Ca^{2+}ATPase homologue suppresses the lethality of *ypt1-1* mutants (Rudolph *et al.*, 1989; see section **8-2b**). Using a cell-free transport system the relationship between Ca^{2+} and Ypt1p was clarified (Baker *et al.*, 1990; Rexach and Schekman, 1991).

The *in vitro*-reaction was shown to consist of two steps. Ypt1p was required for attachment or targeting of vesicles. *ypt1* mutants accumulate vesicles which can fuse when the block is removed (by adding non-mutant cytosol for example). Reactions

blocked by Ca^{2+} chelation accumulate vesicles which are docked onto the Golgi membrane implicating Ca^{2+} in vesicle fusion but not targeting.

7-5 Ca^{2+} and regulated exocytosis

Ca^{2+} influx across the plasma membrane and the release of Ca^{2+} from intracellular stores is responsible for triggering exocytosis of secretory vesicles in many cell types (Burgoyne, 1987). Neurotransmitter release at the presynaptic terminal is also regulated by Ca^{2+} ions. Studies on regulated exocytosis at the synapse have revealed a possible Ca^{2+}-sensitive receptor protein, synaptotagmin, an integral membrane protein found in synaptic vesicles. It has been identified in several species; its gene cloned (Rothblatt *et al.*, 1994) and sequence analysis has revealed two repeats of an amino acid motif called the C2 domain. C2 domains are thought to confer Ca^{2+}-and lipid-binding properties to a large family of proteins (Perrin *et al.*, 1990; Clark *et al.*, 1991). C2-dependent binding of recombinant synaptotagmin to acidic phospholipids has been demonstrated (Perrin *et al.*, 1990) and this binding is enhanced in the presence of Ca^{2+} ions (Brose *et al.*, 1994). Elferink *et al.* (1993) have shown that antibodies against synaptotagmin and recombinant fragments of the protein decrease synaptic vesicle fusion when introduced into PC12 cells. All these observations suggest that synaptotagmin might link Ca^{2+} to regulated exocytosis.

How might synaptotagmin regulate exocytosis? Sollner *et al.*, (1993) have shown that synaptotagmin and α-SNAP share a common binding site in the SNARE complex (see section **5-2** on SNARE hypothesis), and they propose that synaptotagmin might clamp the SNARE complex in a conformation which prevents membrane fusion. An increase in Ca^{2+} concentration would cause synaptotagmin to release the SNARE complex allowing α-SNAP to bind and promote membrane fusion. Ca^{2+} was however found not to promote synaptotagmin release (Sollner *et al.*, 1993) perhaps

indicating it is not a Ca^{2+} sensor. An alternative view is that because of the importance of preventing spontaneous neurotransmitter release other fusion clamps are involved which regulate synaptotagmin release.

8-1 Ca^{2+} homeostasis in yeast

In *S. cerevisiae* the vacuole is the major store of intracellular Ca^{2+}. Eliam *et al.* (1985) showed that most of the cellular Ca^{2+} is found in a compartment which is stable to a treatment with DEAE-dextran. This compartment was shown not to be mitochondria by demonstrating that the intracellular distribution of Ca^{2+} in respiratory-deficient mutants was similar to that of wild type cells. Staining of cells treated with DEAE-dextran with a fluorescent dye revealed that the vacuoles of these cells were intact. This together with the observation that 93% of the vacuolar marker arginine was concentrated in the DEAE-dextran stable fraction indicates that the vacuole is the major Ca^{2+} store.

The cytosolic concentration of Ca^{2+} is maintained at very low levels which have been determined using various Ca^{2+} sensitive fluorescent dyes (Halachmi and Eliam, 1989; Halachmi and Eliam, 1993; Iida *et al.*, 1990). Halachmi and Eliam (1993) used the dye indo-1 to measure both free cytosolic and vacuolar Ca^{2+} concentrations. This dye can permeate through membranes in its acidic form (at ~ pH 4-4.5) but once inside the cells the higher pH prevents crossing of membranes. The dye accumulates in the cytoplasm and not the vacuole, as demonstrated by subcellular fractionation and by measuring the ratio of fluorescence intensities at 410/480 nm. The concentration of free cytosolic Ca^{2+} was estimated at 364 +/- 42 nM. This is similar to other published values (116 +/- 90 nM Iida *et al.*, 1990). To measure the vacuolar Ca^{2+} concentration the cells were treated to lyse the vacuoles and the fluorescence measured; the vacuolar Ca^{2+} concentration was estimated at 1.3 mM.

8-2 Ca^{2+}-sensitive yeast mutants

8-2a *vma* mutants

Calcium transport into the vacuole is thought to be carried out by a Ca^{2+}/ H^{+} antiport system (Ohsumi and Anraku, 1983) although no gene encoding such a protein has been isolated. It is not surprising therefore that mutations in genes which encode subunits of the vacuolar membrane H^{+}ATPase result in Ca^{2+} sensitivity (Ohya *et al.*, 1991). Mutant H^{+}ATPases cannot efficiently maintain the proton motive force necessary to accumulate Ca^{2+} into the vacuole in media containing 100 mM $CaCl_2$. Halachmi and Eliam (1993) have measured the cytosolic Ca^{2+} concentration in *Δvma 4* strains and found that in high Ca^{2+} media the concentration can rise from 116 nM to 4.7 mM. They also showed that if ATP levels were lowered by preincubating cells with metabolic inhibitors, the Ca^{2+} concentration could rise up to 8.8 mM. This indicates that additional ATP-dependent Ca^{2+} transporting mechanisms must be operating in Ca^{2+} homeostasis in yeast.

8-2b Ca^{2+}ATPases

Pmr1p

The *PMR1* gene was cloned by two independent groups (both reported in Rudolph *et al.*, 1989). One group cloned *PMR1* by complementation of a known mutation in the secretory pathway, isolated in a screen for strains which secreted high levels of heterologous proteins. Super-secreting mutants (*ssc*) were isolated using a plate assay screen which detected secreted bovine prochymosin (Smith *et al.*,1985). Mutations in one gene, *SSC1*, could increase secreted levels of heterologous proteins from yeast up to eighty fold. It was proposed that the supersecreting phenotype was due to a bypass of some rate-limiting step in the secretory pathway. The second group cloned the *PMR1* gene by its homology to the yeast plasma membrane ATPase *PMA1* (Serrano *et al.*, 1986). This approach also isolated a related gene, *PMR2*, which also

encodes a P-type ATPase. The *PMR1* gene was predicted to encode a 104 kDa P-type ATPase which resembled Ca^{2+}ATPases found in mammalian cell plasma membranes and sarcoplasmic reticulum. Near its N-terminus Pmr1p has a Ca^{2+}-binding loop similar to that found in Ca^{2+} binding/regulatory proteins such as calmodulin (Davis *et al.*, 1986). Strains carrying a null mutation of *PMR1* have various Ca^{2+} related growth defects. *pmr1* strains grow poorly on low Ca^{2+} media and lose viability in stationary phase more rapidly than *PMR1* strains (Rudolph *et al.*, 1989). *pmr1* strains also show sensitivity to Ca^{2+} chelator EGTA . These defects and sensitivities are all reversible by addition of 20 mM Ca^{2+}. Invertase secreted from *pmr* mutants runs faster on SDS-PAGE gels than that secreted from wild type cells. This is similar to invertase secreted from *mnn9* mutants which are defective in addition of mannose outer chains to glycoproteins (Tsai *et al.*, 1984). As a result of this glycosylation defect, invertase secreted from *pmr1* strains has a more homogeneous appearance than than that from *PMR1* strains. Since *pmr1* mutants had various Ca^{2+}-related phenotypes, the interaction of this allele with *YPT1* was investigated. *ypt1* mutants were thought to be involved in Ca^{2+} balance in yeast (Schmitt *et al.*, 1988). A *pmr1* mutation is able to suppress the temperature sensitive growth phenotype of *ypt1-ts* mutants.

Rudolph *et al.* (1989) suggested that the *pmr1* null mutation might result in a bypass of part or all of the Golgi apparatus and this accounted for the super-secreting phenotype and glycosylation defects. Since then Pmr1p has been localized to the Golgi and proteins secreted from *pmr1* mutants shown to pass through the Golgi (Antebi and Fink, 1992). Passage through the Golgi was demonstrated using antibodies to α1,6-and α1,3-mannose linkages which are markers for early and late Golgi processing (Franzsouff and Schekman, 1989). Invertase secreted by a *pmr1* mutant was shown to have received both modifications. A functional epitope tagged

version of Pmr1p was localized by indirect immunofluorescence and subcellular fractionation. Antebi and Fink (1992) describe a " Golgi-like" distribution because Pmr1p only partly overlaps with known Golgi markers, Kex2p, Sec7p, and GDPase (Redding *et al.*, 1991; Aberijon *et al.*, 1989; Bowser and Novick, 1991). They suggest Pmr1p resides in a different subcompartment of the Golgi from these markers.

Antebi and Fink (1992) also observed that *pmr1* mutants have several other Ca^{2+} -dependent defects. In low Ca^{2+} media proteolytic processing of α-factor is incomplete and this results in secretion of a high molecular weight, highly glycosylated form of α-factor and only a little mature form. This resembles the profile seen in a *kex2* mutant (Julius *et al.*, 1984). *pmr1* strains have a slight mating defect but this and the α-factor processing is complemented by addition of 10 mM Ca^{2+} to the growth media. One explanation for the α-factor processing and glycosylation defects of *pmr1* mutant could be that the enzymes involved have been mislocalized. This could either physically prevent their action or alternatively place enzymes in suboptimal environments reducing their activity. No mislocalization of various organelle markers was detected by Antebi and Fink (1992) although they did notice a small amount of p2CPY was secreted and that this phenotype was reversible by addition of Ca^{2+}.

Finally the interaction of *PMR1* with various *sec* mutants was examined. Deletion of *PMR1* suppressed *sec6*, *sec15*, and *sec19* mutants. Overexpression of Pmr1p resulted in synthetic lethality in *sec21*, *ypt1*, *sec9*, and *sec10* mutants. These observations suggest Ca^{2+} balance has a wide-spread effect on the secretory pathway. Interestingly another Ca^{2+}ATPase activity has been localized to the Golgi in *pmr1* mutants (Okorokov *et al.*, 1993). This activity has been biochemically defined but so far the gene has not been cloned. This discovery raises the possibility of multiple Ca^{2+} ATPases in the secretory pathway.

Pmc1p

The *PMC1* gene was cloned by PCR using oligonucleotides complementary to the conserved regions of previously identified P-type ATPases (*PMA1*, *PMA2*, *PMR1*, *PMR2*) (Cunningham and Fink, 1994). *PMC1* was predicted to encode a 131 kDa Ca^{2+}ATPase and analysis of an epitope-tagged protein by indirect immunofluorescence revealed a predominantly vacuolar distribution. A role for Pmc1p in Ca^{2+} homeostasis was confirmed when a *pmc1* null mutant was constructed. This mutant has no obvious phenotype in standard growth media but in high Ca^{2+} media it accumulates significantly less Ca^{2+} in the vacuole and growth is suppressed. Several unrelated mutations were found to reverse the growth defect of *pmc1* mutants in high Ca^{2+} media. Mutations in the *CNB1* gene which encodes the Ca^{2+} binding regulatory subunit of calcineurin (Cyert and Thorner, 1992) could suppress the *pmc1* phenotype. Deletion of both (but not either one) of the genes encoding the catalytic subunits of calcineurin also suppressed the Ca^{2+} sensitivity of *pmc1* strains. Calcineurin is a Ca^{2+}/calmodulin-dependent phosphoprotein phosphatase (Cyert and Thorner, 1992). Mutant forms of the calmodulin protein (encoded by *CMD1*) which are unable to bind Ca^{2+} fail to activate target enzymes *in vitro* (Hurwitz *et al.*, 1988). Cunningham and Fink (1994) showed that a *pmc1 cmd1-3* double mutant is able to grow well in high Ca^{2+} media. Introducing a wild type calmodulin gene on a low copy number plasmid restored Ca^{2+} sensitivity. In other words the Ca^{2+} sensitive phenotype of *pmc1* mutants requires the activation of calcineurin by calmodulin. Drugs which inhibit calcineurin such as the immunosuppressant drugs FK506 and FKBP-12 are able to suppress the *pmc1* growth defect in high Ca^{2+}. The Pmr1p protein also seems to be important in Ca^{2+} homeostasis and overexpression of Pmr1p in a *pmc1* mutant restores growth in high Ca^{2+} media. *pmc1 pmr1* double mutants are non-viable in all media regardless of external Ca^{2+} levels.

8-2c Csg2p

Recently a screen for mutants that cannot grow in 100 mM Ca^{2+} identified the *CSG2* gene as important for Ca^{2+} regulation (Beeler *et al.*, 1994; Zhao *et al.*, 1994). The sequence of this gene predicts a protein of 45.5 kDa with nine possible transmembrane sequences and a Ca^{2+}-binding domain. Beeler *et al.* (1994) have shown that mutation of this gene alters Ca^{2+}-homeostasis in a non-vacuolar compartment which remains to be identified.

9 Outline of this project

The original aim of my PhD was to investigate ways of overproducing heterologous proteins in yeast. Work on the construction of an episomal expression plasmid for use in the yeast *K. lactis*, and the subsequent expression of human elafin was carried out at ICI Pharmaceuticals, (now Zeneca Pharmaceuticals) and fulfilled this aim. While working at Edinburgh University my interest switched to Golgi structure and function, particularly the effects of the *pmr1* null mutation. The *pmr1* mutant had originally been of interest because of its reported supersecreting phenotype.

Chapter 2
Materials and Methods

2 Materials

2-1 Chemicals, enzymes, and antibodies

Most chemicals were obtained from BDH Chemicals and the Sigma Chemical Company. Restriction and DNA modifying enzymes were purchased from Promega, NEB, Amersham, and Gibco BRL. Taq polymerase was from Promega and Vent Polymerase from NEB. The USB Sequenase version 2.0 kit and the CircumVent Thermal Cycle DNA sequncing kit from NEB were used for DNA sequencing. The α-^{35}S-dATP for DNA sequencing was from Amersham and NEM DuPont. Hybaid supplied premade DNA sequencing gel solutions. HRP antibody conjugates were from either the Scottish Antibody Production Unit or from Sigma. Hybond N+, Hybond C-extra, and the Enhanced Chemilumonescence (ECL) Detection kit were supplied by Amersham. Pansorbin for the immunoisolation experiments was from Calbiochem. The synthetic peptide (b-QRR MCA) for the Kex2p assay was from the Peptide Institute Inc., Japan. Zymolyase for spheroplasting was from the Seikagaku Kogyo Co., Japan. Pharmacia LKB supplied the IgG-Sepharose (Fast Flow). Affi-gel and poly prep columns were from Bio Rad. Media components were from Difco Laboratories.

2-2 Bacterial and Yeast strains

Strains of *E. coli*, *S. cereivisiae*, and *K. lactis* used in this project are listed in table 1 (see appendix). Transformants are denoted in the text by listing the strain, followed by the plasmid with which it has been transformed given in parenthesis.

2-3 Media

Bacterial cultures were grown in Luria broth containing 1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, and 0.5% (w/v) NaCl. Cultures were grown at 37^{o}C. Antibiotics were added as required (ampicillin at 100 μg ml^{-1} and kanamycin at

80 μg ml^{-1}). 2% Bacto agar was added to make plates. Yeast cultures were grown in: complete media (YPD) containing 1% (w/v) Bacto yeast extract, 2% (w/v) Bacto peptone, and 2% (w/v) glucose. Yeast cultures were grown at 30°C. YPG and YPL were similar but contained 2% (w/v) galactose or lactose respectively; minimal (selective) media (SD) containing 0.67% Bacto yeast nitrogen base without amino acids and 2% (w/v) glucose. The following were added to SD as required: histidine (20 μg ml^{-1}), leucine (30 μg ml^{-1}), tryptophan (20 μg ml^{-1}), uracil (20 μg ml^{-1}), lysine (30 μg ml^{-1}), arginine (20 μg ml^{-1}). 2% Bacto agar was added to yeast media to make plates.

Methods

2-4 Bacterial transformation

Competent *E. coli* were prepared as follows, using a modification of the protocol described in Sambrook *et al.*, 1989. A fresh overnight culture was grown in 5 ml of LB broth at 37°C. This culture was used to inoculate a flask, containing 100 ml of LB broth, to an OD_{600} of about 0.1. The flask was shaken at 37°C until the OD_{600} reached 0.4- 0.7. On reaching this OD_{600} the culture was chilled by swirling in an ice-water bath for several minutes. The chilled culture was centrifuged for 5 min, 3000 g, at 4°C. The supernatant was removed and 40 ml of ice-cold $CaCl_2$ added. The cells were gently resuspended and then placed on ice for at least 30 min, with occasional swirling. Next the cells were centrifuged for 5 min, 3000 g, at 4°C and then resuspended in 5 ml of ice cold 100 mM $CaCl_2$, containing 15% glycerol. This suspension was aliquoted into 200 μl amounts in prechilled microcentrifuge tubes. These were left overnight on ice and then the next morning placed in a -70°C freezer. The highly competent cells produced remained competent for several months when prepared and stored in this way.

Frozen competent cells were defrosted slowly on ice before transformation. Plasmid DNA or ligation mixture was added to the cells, gently mixed, and then left on ice for at least 30 min. The cells were then heat shocked for 3 min at 42°C, followed by 3 min on ice. Next 800 µl of LB broth was added and the cultures shaken for at least 40 min at 37°C to allow expression of antibiotic resistance genes. After this the cells were plated out at various dilutions.

2-5 Yeast transformation

This protocol is a modification of one published by Gietz *et al.*, (1992). Cells were inoculated into YPD medium and grown overnight at 30°C. Next morning cells were diluted in 50 ml of YPD to an OD_{600} of ~0.1. Cultures were grown to an OD_{600} of between 0.5-1.0 and then centrifuged at 3000 g, for 5 min at room temperature or 4°C. Harvested cells were washed once in TE (10 mM Tris-HCl, 1 mM EDTA, pH7.5) and then resuspended in 1 ml of TE/lithium acetate (TE containing 100 mM LiAc). To 200 µl of cells, in a microcentrifuge tube, plasmid in TE (usually 10-20 µg) was added. Next 300 µl of 40% PEG 4000 solution (40% PEG 4000, 10 mM Tris-HCl, 1 mM EDTA, 100 mM LiAc, PH 7.5) was added and gently mixed. This mixture was incubated with gentle shaking at 30°C for 30 min. After this the cells were heat shocked for 15 min at 42°C, spun down and washed in TE. The cells were then resuspended in 200 µl of TE and plated onto selective media.

2-6 DNA preparation and Manipulation

Most DNA manipulations were as outlined in Sambrook *et al.* (1989). Plasmid DNA was prepared using Qiagen columns and Promega Magic Mini Prep columns according to manufacturers' instructions. Mini-preps were also carried out using the following "quick and dirty" protocol. 1.5 ml of an overnight bacterial culture grown at 37°C was spun for 1 min at 20 000 g. The supernatant was removed and the cells

resuspended in 200 µl of solution 1 (25 mM Tris.HCl pH 8.0, 50 mM glucose, 10 mM EDTA). Before addition of solution 1 to cells a small spatula end of lysozyme was added per 5 ml. After resuspension the cells were incubated for 5 min at room temperature. Next 400 µl of solution 2 was added (0.2 M NaOH, 1% SDS), the microcentrifuge tube inverted gently 6 times and incubated for 5 min at room temperature. After this the suspension clears and becomes viscous. 300 µl of 3M sodium acetate, pH 4.8 was added and after several gentle inversions the microcentrifuge tube was placed on ice for 5 min. A white precipitate forms which was spun down (10 min, 20 000 g). 750 µl of the supernatant was carefully removed and placed in a fresh microcentrifuge tube. To this 500 µl of isopropanol was added and after gentle mixing by inversion the precipitated DNA was spun down (20 000 g, 5 min). The isopropanol was removed (it is not important to dry the tube completely) and 200 µl of TE (10 mM Tris.HCl, pH 8, 1 mM EDTA) added. To this was added 100 µl of phenol equilibrated to pH 8.0 with Tris.HCl and 100 µl of chloroform/isoamyl alcohol (24:1). The tube was then vortexed for 30 s. After vortexing the tube was spun (20 000 g, 3 min) and the top aqueous phase removed carefully (about 200 µl), avoiding the white precipitate at the interface with the phenol/chloroform/isoamyl alcohol. The DNA in the aqueous phase was precipitated by addition of 20 µl of sodium acetate, pH 4.8, and 500 µl of ethanol. After 30 min at -70 ^{o}C the DNA was spun down (20 000 g, 10 min) and the ethanol removed. The microcentrifuge tubes were dried (upside down beside a lit bunsen burner) to remove any residual ethanol. The DNA pellet was resuspended in 50 µl of TE. 5 µl usually contained about 1 µg of DNA (high copy number plasmid). DNA prepared this way was used as template for DNA sequencing but for much better results DNA prepared using commercial kits was used.

Restriction digests were usually carried out in a volume of 50 µl using 10 units of each

enzyme, and incubated for 1-4 hours. Gel electrophoresis used for the separation of DNA fragments was carried out using 0.6-2.0% agarose gels. TAE buffer containing 0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0 was used. Sizes of fragments were estimated by comparison of their mobility through the gel with fragments of known size (1 kb ladder, Gibco BRL).

2-7 Preparation of yeast genomic DNA

5 ml cultures of yeast were grown overnight in YPD or SD at 30°C. Cells were spun down (20 000 g, 30 s) in a microcentrifuge tube, and the pellet resuspended in 200 µl of a solution containing: 2% Triton X100, 1% SDS, 100 mM NaCl, 10 mM Tris.HCl (pH 8.0), 1 mM EDTA. 100 µl of phenol and 100 µl of a chloroform/isoamyl solution (24:1) was added along with 0.3 g of glass beads (40 mesh). The microcentrifuge tube was then vortexed for 2 mins and spun for 2 min at 20 000 g. The upper aqueous phase was removed, being careful not to disturb the interface. This fraction was ethanol precipitated by addition of 1/10 vol of 3 M NaOAc (pH 5.2) and 2.5 vols of ethanol. The precipitated DNA was spun down (20 000 g, 5 min) and resuspended in 50 µl of TE buffer (10 mM Tris.HCl (pH 8,0), 1 mM EDTA). DNA prepared in this way was suitable for restriction digest prior to Southern blotting and as a template for PCR.

2-8 Southern blotting

After electrophoresis of restricted yeast genomic DNA in TAE agarose gels, DNA was transferred by capillary action to Hybond N+ (a positively charged nylon filter). The gel was processed as described in the Hybond N+ instruction booklet and a capillary blot was set up (also described in the Hybond N+ instructions). After overnight transfer DNA was crosslinked to the filter by UV radiation in a Stratalinker (Stratagene). The moist filter (not dripping wet) was placed on 3MM paper in the

Stratalinker and autocrosslink mode used. Detection of DNA was performed using the Boehringer Mannheim DIG system. DIG probes were labelled by PCR by spiking a standard PCR mixture with dig-dUTP (8 µl dNTPs mix containing 1.25 mM of each of dATP, dCTP, dGTP, and dTTP/dig-dUTP in a ratio of 9:1). The Lumigen PDD chemiluminescent detection protocol was followed as per manufacturers' instructions (Boehringer Mannheim except for the incubation step with Lumigen PDD). 1 ml of a 1:100 dilution of the stock Lumigen PDD was made. After equilibration in buffer 3, the filter was placed on a piece of Saran Wrap and the 1 ml of diluted Lumigen PDD was pipetted onto the filter at several locations. Another piece of Saran Wrap was then placed on top of the filter causing the Lumigen PDD to spread out evenly over the filter. After a 5 min incubation the Saran Wrap was removed and the filter blotted on 3MM paper for a few seconds. The filter was then wrapped in fresh Saran Wrap and incubated for 10 min at 37°C before X-ray film was layed down. This procedure saves greatly on reagent and does not effect sensitivity.

2-9 DNA sequencing

All DNA sequencing used double-stranded DNA (prepared using Qiagen plasmid preparation columns, or Promega Magic DNA columns) as template. The method of Hsaiao (1991) using the Sequenase version 2.0 kit was used. Some screening for recombinant clones was performed using the CircumVent Thermal Cycle DNA sequencing kit (NEB). 6% polyacrylamide, wedge gels were used to maximise the number of readable bases from a single run. Premade gel solutions were used (Hybaid).

2-10 Polymerase Chain Reaction (PCR)

PCR extension was carried out using either Taq or Vent Polymerase. PCR was carried out in 100 µl reactions according to manufacturer's instructions. The number of cycles and extension times were minimized to reduce the chance of PCR errors occuring. Screening for recombinant plasmids in bacterial culture/colonies was performed by PCR as follows. Either half a colony or 5 µl of an overnight culture was inoculated directly into a standard 100 µl PCR mixture containing appropriate diagnostic oligonucleotides. The heat from the first few cycles lyses the cells releasing plasmid DNA which acts as template. After 20-30 cycles 20 µl of the reaction mixture was examined by gel electrophoresis for product of the correct size. Care was taken to avoid cross contamination of samples and a control containing no template DNA was always performed. If possible a positive control was also performed.

2-11 Gel purification of DNA fragments

DNA fragments generated by restriction digest and PCR products often had to be purified from TAE agarose gels. This was done using either the GeneClean or Qiaex kits. Manufacturers instructions were followed and although recovery was good in both cases the Qiaex was more reliable in my hands.

2-12 Cloning PCR products

PCR products were cloned in several ways, usually after gel purification. Products generated by Taq polymerase have overhanging ends consisting of dATP added by the terminal transferase activity of Taq. This allowed cloning into T-vectors (Promega and InVitrogen) which have overhanging T residues which can be ligated to the terminal A residues of the products. Vent Polymerase has no terminal transferase activity and this means products can be cloned directly as blunt fragments. An alternative cloning procedure was to purify the product then digest it with appropriate

enzymes and clone into a vector similarly digested.

2-13 Oligonucleotide-directed *in vitro* mutagenesis

Two methods were used, the method of Kunkel *et al.* (1987) and the method of Eckstein *et al.* (1988). The Kunkel method selects against the non-mutagenized strand on the basis of uracil-containing DNA being selected against. DNA to be mutagenized was cloned into M13mp18. The replicating form (RF) of M13mp18 was synthesized in *E. coli* strain BW313 which is a *dut ung* double mutant. DNA synthesized in this strain contains uracil in some thymine positions as a result of the *dut* mutation which inactivates dUTPase. This results in high intracellular levels of uracil. The *ung* mutation inactivates uracil N-glycosylase allowing incorporated uracil to remain in the DNA. RF DNA was isolated from BW313 and used in an *in vitro* synthesis reaction which included the appropriate mutagenic oligonucleotide as a primer. When transformed into a strain (NM522) with an active uracil N-glycosylase only non-uracil containing DNA survives and is replicated. The presence of the correct mutation was confirmed by DNA sequencing.

In the Ekstein method (1988) removal of the non-mutant strand is made possible by the incorporation of a thionucleotide into the mutant strand during the *in vitro* synthesis reaction. Certain restriction enzymes cannot cut DNA containing this thionucleotide. Restriction with such an enzyme results in single strand nicks which allows exonuclease III to bind and digest away the non-mutant strand. The Amersham Oligonucleotide-directed *in vitro* mutagenesis system version 2.1 kit was used which is based on this method. Manufacturer's instructions were followed.

2-14 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out basically as described by Laemmli (1970) using the following solutions:

Separating gel buffer 0.75 M Tris.HCl (pH 8.8), 0.2% (w/v) SDS

Stacking gel buffer 0.25 M Tris.HCl (pH 6.8), 0.2% (w/v) SDS

Acrylamide solution 44% (w/v) acrylamide, 0.8% (w/v) N N'-methylene bisacrylamide

Electrophoresis buffer 0.125 M Tris.HCl (pH 8.3 without adjustment), 0.1% (w/v) SDS

9-12% (w/v) separating gels were run with a 5% (w/v) stacking gel. Samples were resuspended in SDS-sample buffer containing 8 M urea :

0.0625 M Tris.HCl (pH 6.8), 20% (w/v) glycerol, 4% (w/v) SDS,

5% (w/v) β-mercaptoethanol, 8 M urea.

2-15 Semi-dry Western blotting

Western blotting was performed using a Pharmacia LKB semi-dry blotter. Six pieces of 3MM paper and one piece of Hybond-C extra (supported nitrocellulose) were cut to the size of the gel to be blotted. Two pieces of 3MM paper were soaked in anode buffer 1, one piece in anode buffer 2, and three pieces in cathode buffer. The gel was also soaked in cathode buffer and the Hybond-C extra wetted in distilled water. The paper, Hybond-C extra, and gel were assembled on the anode plate as follows:

First 2 pieces 3MM paper (anode buffer one)
1 piece 3MM paper (anode buffer two)
Hybond-C extra (water)
gel (cathode buffer)

Last 3 pieces 3MM paper (cathode buffer)

The cathode plate was placed on top of the sandwich and transfer achieved by applying a current of 0.8 mA per cm^2 gel area. Composition of the buffers is listed below:
Anode buffer 1: 0.3M Tris.HCl (pH 10.4), 20% (w/v) methanol, 0.1% (w/v) SDS
Anode buffer 2: 25 mM Tris.HCl (pH 10.4), 20% (w/v) methanol, 0.1% (w/v) SDS

Cathode buffer: 25 mM Tris.HCl (pH 9.4), 20% (w/v) methanol, 0.1% (w/v) SDS
40 mM 6-amino-n-hexanoic acid

2-16 ECL detection of blotted proteins

Nitrocellulose filters onto which proteins had been western blotted were incubated in blocking buffer (5% (w/v) non-fat dried milk (Marvel), 1% (v/v) Tween-20 in TBS; 10 mM Tris.HCl (pH 7.4), 150 mM NaCl) to block unfilled sites. Filters were gently agitated in blocking buffer for a minimum of 1 hour (at room temperature), overnight being more usual. Primary antibody was diluted in blocking buffer and incubated with the filter for 1 hour, again with gentle agitation. Unbound primary antibody was washed away with vigorous agitation by one 10 min wash followed by three 5 min washes in 1% (v/v) Tween-20 in TBS (washing buffer). If a second antibody was used it was diluted in washing buffer and incubated with the filter with gentle agitation for 30 min to 1hour. Unbound second antibody was washed away as for primary antibody. Western blots were developed using enhanced chemiluminesence (ECL) with ECL reagents as described in the manufacturer's instructions.

2-17 Affinity-purification of anti-Kex2p antibodies

2 ml (slurry volume) of Affi-gel 10 was washed once in 10 ml of water and once in 10 ml of 0.1 M Hepes (pH 7.4). The Affi-gel was allowed to settle by gravity on ice between all washes. Approximately 500mg of a βgal-Kex2p fusion protein (kindly supplied by Dr. N. Bryant; Bryant and Boyd, 1993) was resuspended in 10 ml of 0.1 M Hepes (pH 7.4) and bound to the Affi-gel by allowing gentle mixing on a rotating wheel overnight at 4°C. Unbound protein was washed away with 0.1 M Hepes (pH 7.4). Washing continued until the A_{280} of the buffer was close to zero. Remaining reactive groups on the Affi-gel were quenched by incubation with 10 ml of 0.2 M glycine.HCl (pH 8.0) for 1 hour at 4°C. The Affi-gel was then washed twice with 0.1 M Hepes (pH 7.4) and once with 0.2 M glycine.HCl (pH 2.8) for 5 min at room temperature. The pH was restored to pH 7.4 by washing with 0.1 M Hepes (pH 7.4) before the Affi-gel affinity matrix was incubated for 2 hours with 10 ml of immune serum from a rabbit injected with a Kex2p-protein A fusion protein (Bryant and Boyd, 1993). The affinity matrix was then packed in a 10 ml Poly Prep chromatography column. The column was washed with 0.1 M Hepes (pH 7.4) until the buffer coming through had a A_{280} close to zero. Antibodies were eluted by washing with 0.2 M glycine.HCl pH 2.8. 1 ml fractions were collected in microcentrifuge tubes containing 75µl of 1 M Tris.HCl (pH 8.0). The first four fractions contained most of the antibody and these were pooled. Sodium azide was added to 0.1% before storage at -70°C.

2-18 Yeast cell glass bead lysate

Cultures were grown to an OD_{600} of between 0.6 and 1.0 and then cells spun down (3 000 g, 5 min). The pellet was washed once in lysis buffer (100 mM Tris.HCl, 1 mM EDTA) before being resuspended to about 100 OD_{600} units ml^{-1} in lysis buffer containing protease inhibitors (1 mM PMSF, 0.4 mg ml^{-1} each of pepstatin,

leupeptin, chymostatin, and antipain, 1 mM EDTA, 1 mM EGTA). The resuspended pellet was transferred to the required number of microcentrifuge tubes and glass beads (40 mesh) added up to the level of the meniscus. The tubes were vortexed for four 30 s bursts with 1 min on ice between each burst. After vortexing a hot needle was used to make a small hole in the lid and base of the mictocentrifuge tube. The microcentrifuge tube was then placed in a 10 ml Falcon tube and spun for 3 000 g, 5 min. The yeast extract passes into the Falcon tube, the glass beads remain behind in the eppendorf. A pellet of unbroken cells and cell debris forms covered by yeast cell lysate.

2-19 Yeast cell homogenate

Cells were harvested (3 000 g, 5 min) washed once in 10 ml of water and resuspended at 100 OD_{600} units ml^{-1} in spheroplast buffer (1.4 M sorbitol, 50 mM Tris.HCl (pH 7.5, 10 mM NaN_3, 40 mM β mercaptoethanol). 5 mg of zymolyase was added and spheroplasting achieved by incubation for 30 min at 30°C with occasional shaking. The efficiency of spheroplast conversion was checked by removing two 10 µl samples. One was diluted in spheroplast buffer and the other in distilled water. The OD_{600} of the two diluted samples were measured and the proportion of cells spheroplasted calculated as follows:

$$\frac{OD_{600} \text{ in spheroplast buffer} - OD_{600} \text{ in distilled water}}{OD_{600} \text{ in spheroplast buffer}}$$

Once >90% of cells were converted to spheroplasts they were harvested (500 g, 5 min) and washed once in spheroplast buffer. The spheroplasts were then resuspended in homogenization buffer (100 OD_{600} units ml^{-1}) containing protease inhibitors (0.8 M sorbitol, 10 mM triehanolamine, 1 mM EDTA, brought to pH 7.4 with acetic

acid and containing protease inhibitors as above). The spheroplasts were homogenised with 10 strokes in a dounce homogenizer on ice. The homogenate was cleared before use in immunoisolation procedures by a spin at 4 000 g, 5 min.

2-20 Immunoisolation procedures

The method developed by Bryant and Boyd (1993) and Bryant (1992) were used with only slight modifications, outlined below.

2-20a Preparation of Kex2-Immunoadsorbent (ImAd)

All steps described here were carried out at 4°C and after washes and incubations Pansorbin cells were spun down for 2 min at 4000 g. Pansorbin cells were washed in binding buffer (20 mM Hepes pH 7.2, 2 mM $MgCl_2$, 150 mM KCl) and then resuspended in blocking buffer (binding buffer containing 10 mg ml^{-1} bovine serum albumin), using 1 ml / 50µl original 10% (w/v) cell suspension. The microcentrifuge tubes were then placed on a rotating wheel for 1 hour. The blocked cells were washed for 15 min in binding buffer before addition of affinity-purified anti-Kex2p antibody. For the production of the standard ImAd used, 1 µl of affinity-purified antibody (5 µg protein) was added for each µl of cell suspension and the sample volume made up to 500 µl with binding buffer. After incubation on a rotating wheel for 3 hours or overnight the ImAd was washed once (15 min) in binding buffer and once in homogenization buffer (see section **2-18**)

2-20b Immunoisolation of Kex2p-containing organelles

ImAd was resuspended with 300 µl (approximately 300 µg protein) of fresh yeast cell homogenate as described in section **2-18**. This was left to incubate on a rotating wheel for 2-3 hours. The ImAd was recovered by centrifugation (4000 g, 2 min) and washed in homogenization buffer once for 5 min on a rotating wheel (at 4°C), then 4

times by simple resuspension, followed by another 5 min wash on the wheel. The ImAd and recovered material were either used immediately for various enzyme assays or stored at -20°C overnight.

After recovery of Kex2p containing vesicles the immunoadsorbant was washed in homogenisation buffer once for 5 min on a rotating wheel (at 4°C), then 4 times by simple resuspension followed by another 5 min wash on the wheel. When IgG-Sepharose was used to isolate protein A tagged Pmr1p the same protocol as used for Kex2p-protein A isolation was used as outlined in Bryant (1992) except that a blocking step was introduced. This involved incubation of the IgG-Sepharose with BSA (10 $mgml^{-1}$ in binding buffer) for 1 hour at 4°C on a rotating wheel. Without this step a high degree of non-specific binding was observed which was not reported by Bryant (1992). Washing steps were also modified as described above.

2-21 Enzyme assays

2-21a Kex2p activity assay

The sample being assayed for Kex2p protease activity was added to 50 µl Kex2p-assay mix (200 mM Hepes pH 7.0, 1 mM $CaCl_2$, 0.5 mM PMSF, 0.1 mM L-1-tosylamido-2-phenyl-ethyl chloromethyl ketone, 1% (w/v) Triton-X-100, 100 mM *t*-butoxycarbonyl-Gln-Arg-Arg-4-methylcoumarin-7-amide (bQRR-MCA)). Reaction mixtures were incubated at 37°C for 30 min after which time the reaction was terminated by the addition of 0.9 ml 125 mM $ZnSO_4$ and 0.1 ml of a saturated $Ba(OH)_2$ solution. The precipitate formed upon addition of $Ba(OH)_2$ was removed by centrifugation in a microcentrifuge tube (20 000 g , 1 min). The amount of Kex2p protease activity in the sample was determined by detecting the free 7-amino-4-methylcoumarin (AMC) released from bQRR-AMC following cleavage by Kex2p (after the pair of arginine residues). The amount of AMC in the sample was

determined fluorimetrically, λ(excitation)= 385 nm, λ (emission)= 465 nm. Assay results are expressed in arbitrary units of fluoresence.

2-21b Carboxypeptidase Y (CPY) assay

50 µl of the sample to be assayed was added to 1 ml of assay mix containing 0.125 mg ml^{-1} L-amino acid oxidase, 0.2 mg ml^{-1} peroxidase, 0.5 mM $MnCl_2$, 0.1 mg ml^{-1} dianisidine.HCl, and 5 mM peptide substrate (*N*-carbobenzoxy-L-phe-L-leu) in 0.1 M potassium phosphate buffer (pH 7.0). The complete mixture was incubated at 37°C for 90 min after which time the A_{405} of the mix was read against a blank reaction mix that had been incubated with 50 µl of distilled water.

2-21c Dipeptidyl aminopeptidase (DPAP) assays

Total DPAP activity in a sample was assayed by adding the sample, in a 200 µl volume, to 250 µl 400 mM Tris.Hepes (pH 7.0) and 50 µl of a 3 mM solution in 25% methanol of the substrate X-pro-pNA and incubated at 37°C for 30 min. The reaction was stopped by the addition of 500 µl $ZnSO_4$ and 100 µl 7.5% $Ba(OH)_2$. This led to the formation of a precipitate which was removed by centrifugation (20 000 g, 1 min). The A_{405} of the supernatant was read against a blank to which 200 µl of distilled water had been added in place of sample. The activity of DPAP A (which is heat stable) was assayed by heating the sample to 65°C for 30 min prior to its addition to the assay (being allowed to cool to 37°C following heat treatment). The activity of DPAP B was estimated by subtracting the amount of heat stable DPAP activity in the sample from the total amount of DPAP activity in the sample.

2-21d NADPH cytochrome c oxidoreductase assay

Two identical aliquots of the sample to be assayed were each added, in 20 µl volumes to 970 µl of reaction mix containing 1.5 mg ml^{-1} cytochrome c in 300 mM potassium

phosphate buffer (pH 7.4) in matched quartz cuvettes. 10 µl of NADPH (16 mg ml^{-1} in the phosphate buffer) was added to one of these samples and the change in A_{550} was followed.

2-22 Protein concentration determination

Protein concentration was determined using the bicinahonic acid (BCA) protein assay reagent kit (Pierce). This kit is based on the biuret reaction (protein reducing Cu^{2+} in an alkaline medium to Cu^{1+}). The cuprous ions (Cu^{1+}) interact with the BCA giving a soluble purple reaction product which absorbs at 562 nm. Kit instructions were followed.

2-23 Subcellular fractionation by sucrose gradient

Yeast cell homogenates were prepared from 500 OD_{600} units of culture as described above with two modifications: 1) cells were resuspended at 500 OD_{600} units ml^{-1} 2) lysis buffer contained 20 mM triethanolamine (pH 7.2), 12.5% (w/v) sucrose, and 1 mM EDTA, plus protease inhibitors. After homogenisation, a clearing spin was performed (500 g, 5 min) before the homogenate was layered onto a gradient containing 1 ml steps of 18, 22, 26, 30, 34, 38, 42, 46, 50, and 54% sucrose (w/w or w/v) in 10 mM Hepes (pH 7.5), 1 mM $MgCl_2$. Gradients were spun either for 2.5 hours at 174 000 g or overnight in a Beckman SW41 rotor at 4°C. Gradients were fractionated from top to bottom using a 1 ml pipette. Fractions to be analysed by SDS-PAGE were treated as follows. The fraction was diluted 1 in 2 with distilled water and made to 10% (w/v) TCA concentration. Samples were left on ice for at least 1 hour and the precipitate spun down (20 000 g, 5 min). The white sticky precipitates were resuspended in SDS-sample buffer often with several hours incubation at 75°C. Pellets were often difficult to resuspend because of sucrose precipitation.

2-24 Elafin assay

A 0.1 M stock solution of the synthetic tetrapeptide elastase substrate methoxy-succinyl-alanyl-alanyl-propyl-valyl-p-nitroanilide was made in DMSO. This is stable for several months at 4°C. The assay buffer contains 0.1 M Hepes, 0.5 m NaCl (pH 7.5), and 1% (w/v) BSA (high purity since it has to be free of α1 antitrypsin). A stock solution of porcine elastase (1 mg ml^{-1} in 0.1 M NaOAc, 1 M NaCl pH 6.0) is also prepared. Substrate is diluted 40 fold with assay buffer to give a working solution. Samples are also diluted in assay buffer if required. The spectrophotometer is zeroed with 2 samples containing 200 µl assay buffer and 800 µl substrate solution. 100 µl of samples containing purified human elafin (for the standard curve) or 100 µl samples of yeast culture supernatants are incubated with 100 µl of porcine elastase enzyme and incubated for 30 mins. After this 800 µl of substrate is added and the A_{405} monitored for 2 mins. The maximum OD_{405} is noted. This was done twice for each sample. A control with 200µl buffer of yeast supernatant from a strain not secreting elafin was always performed. Elastase activity was calculated as below:

	first reading	second reading	average
control no elafin	A	B	$\frac{A+B}{2}=y$
elafin	α	β	$\frac{\alpha+\beta}{2}=x$

$$\frac{x}{y} \times 100 = \text{elastase activity}$$

Chapter 3
Effects of the *pmr1* mutation on Golgi organization

3-1 Introduction

As previously discussed, loss of the putative Ca^{2+}ATPase Pmr1p results in a pleiotropic phenotype in low Ca^{2+} medium. Many of these effects are reversible by adding Ca^{2+} into the growth media. Rudolph *et al.* (1989) proposed that the phenotype may be due to a bypass of part or all of the Golgi apparatus. Perhaps some proteins in a *pmr1* mutant follow an alternative secretory pathway. Other such pathways are known to exist. For example, secretion of the yeast mating pheromone **a** factor, a farnesylated dodecapeptide, is dependent on the *STE6* gene product. Ste6p is a member of the ABC-transporter family, and is a multispanning plasma membrane protein. This pheromone does not enter the secretory pathway but is secreted by the Ste6p pump in an ATP-dependant process (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989). Maybe loss of Pmr1p results in some proteins being exported in a manner similar to **a** factor, by Ste6p or some similar pump. In human-activated macrophages two types of interleukin-1 (IL1-α and IL1-β) lack a secretion signal. Their export does not occur via the ER and the Golgi apparatus (Hazuda *et al.*, 1988; Young *et al.*, 1988). This chapter describes two lines of work carried out to shed light on the nature of the *pmr1* mutation. Firstly the possibility of a bypass of the Golgi was investigated. After publication of two papers (Antebi and Fink, 1992; Harmsen *et al.*, 1993) and results presented below (ruling out a bypass), attention was switched to Golgi organisation.

3-2 Construction of a strain carrying a null mutation of *PMR1*

The plasmid pHR69 is plasmid pUC19 carrying the 1.7 kb *Hin*dIII fragment of *PMR1* into which a functional *URA3* gene has been inserted (Rudolph *et al* 1989). This plasmid was digested with *Hin*dIII and the 2.8kb *Hin*dIII fragment was gel-purified. This fragment was used to introduce a *pmr1-2::URA3* disruption into yeast strain JRY188 by transformation. Ura^+ transformants were selected and should have had

their chromosomal copy of the *PMR1* gene replaced with the *pmr1-2::URA3* disruption. The *pmr1-2::URA3* disruption was confirmed by Southern blot analysis (**FIG 3-1a, and FIG 3-1b**). Transformants 1, 3, and 4 (see lanes 2, 4, and 5) have had their chromosomal *PMR1* gene replaced by *pmr1-2::URA3*. The JRY188 *pmr1-2::URA3* disruptant was called ACY100. This disruption is in a sequence which encodes for a part of Pmr1p thought to be involved in ATP binding (Rudolph *et al.*, 1989). Four predicted transmembrane domains are also removed. However it is important to note that the 5' portion of the *PMR1* gene is intact and could potentially encode a protein consisting of 682 amino acids from Pmr1p (size ~ 75 kDa). It is possible that if this protein is expressed it could still perform some of the fuctions of an intact Pmr1p.

3-3 ACY100 is sensitive to EGTA

It has been reported that strains carrying a null mutation of *PMR1* are sensitive to EGTA (Rudolph et al 1989, Antebi and Fink 1992). ACY100 and JRY188 grew at similar rates on YPD, a low Ca^{2+} medium (Antebi and Fink, 1992; Ohya *et al.*, 1984) (see **FIG 3-2a**). However when JRY188 and ACY100 were plated on a low Ca^{2+} media (YPD) containing 25 mM EGTA both strains grew slowly but eventually JRY188 has a normal appearance whereas the null mutant is sick (**see FIG 3-2b**).

3-4 The phenotype of *pmr1* null mutants is not due to a bypass of the Golgi

To demonstrate whether proteins secreted by *pmr1* null mutants passed through the Golgi or not, processing by the Kex2p protease was monitored. The plasmid pYJS50 carries the *E. coli* β-lactamase gene fused to the α-mating factor secretion signal. Processing of this signal relies upon the activity of Kex2p, an endoprotease found in a late Golgi compartment. ACY100 secretes processed β-lactamase (**FIG 3-3**)

FIGURE 3-1 Southern blot analysis of potential *pmr1-2::URA3* disruptants.

a) Genomic DNA was prepared from JRY188 and 4 potential disruptants. Samples of this DNA were digested with *Hin*dIII and run out on a 0.8% TAE agarose gel. After Southern blotting the filter was probed with a 1.7 kb DIG-dUTP labelled probe (described below). This probe will hybridize to a 1.7 kb *Hin*dIII fragment from JRY188 or undisrupted transformants, and to a 2.8 kb *Hin*dIII fragment from disrupted strains. DNA detection was performed as described in the Boehringer Lumigen PDD instructions.

DIG-labelled probe was prepared as follows. Plasmid pL113-3 carries an 8.1kb *Aat*II fragment containing the complete *PMR1* gene. This plasmid was digested with *Hin*dIII and the 1.7 kb HindIII fragment cloned into the *Hin*dIII site of pGEM7Zf(+) (Promega). DIG-labelled probe was produced by performing a PCR reaction spiked with DIG-dUTP using this plasmid as target DNA. Primers used were standard forward and reverse sequencing primers. PCR cycle conditions were 95°C 1 min, 55°C 1 min, 72°C 2 min, for 30 cycles and Taq polymerase (Promega) was used. A 10 µl aliquot was removed from the completed PCR reaction to check the reaction was successful. A single band in the correct size range (1.7 kb) was produced. 5 µl of unpurified probe was used in the hybridization buffer. **Lane 1**: genomic DNA from JRY188. **Lanes 2-5**: genomic DNA from 4 potential *pmr1-2::URA3* disruptants.

b) This figure shows a schematic of the genomic DNA from a *PMR1* strain and a *pmr1::URA3* strain. Plasmid pHR69 carries the 1.7 kb *Hin*dIII (H) fragment from *PMR1* with the *URA3* gene cloned into the *HpaI* (Hp) site which is destroyed in cloning. This fragment was used to produce *pmr1::URA3* disruptants. The probe described above is made from the 1.7 kb fragment and will hybridize to the 1.7 kb and the 2.8 kb fragments indicated by arrows.

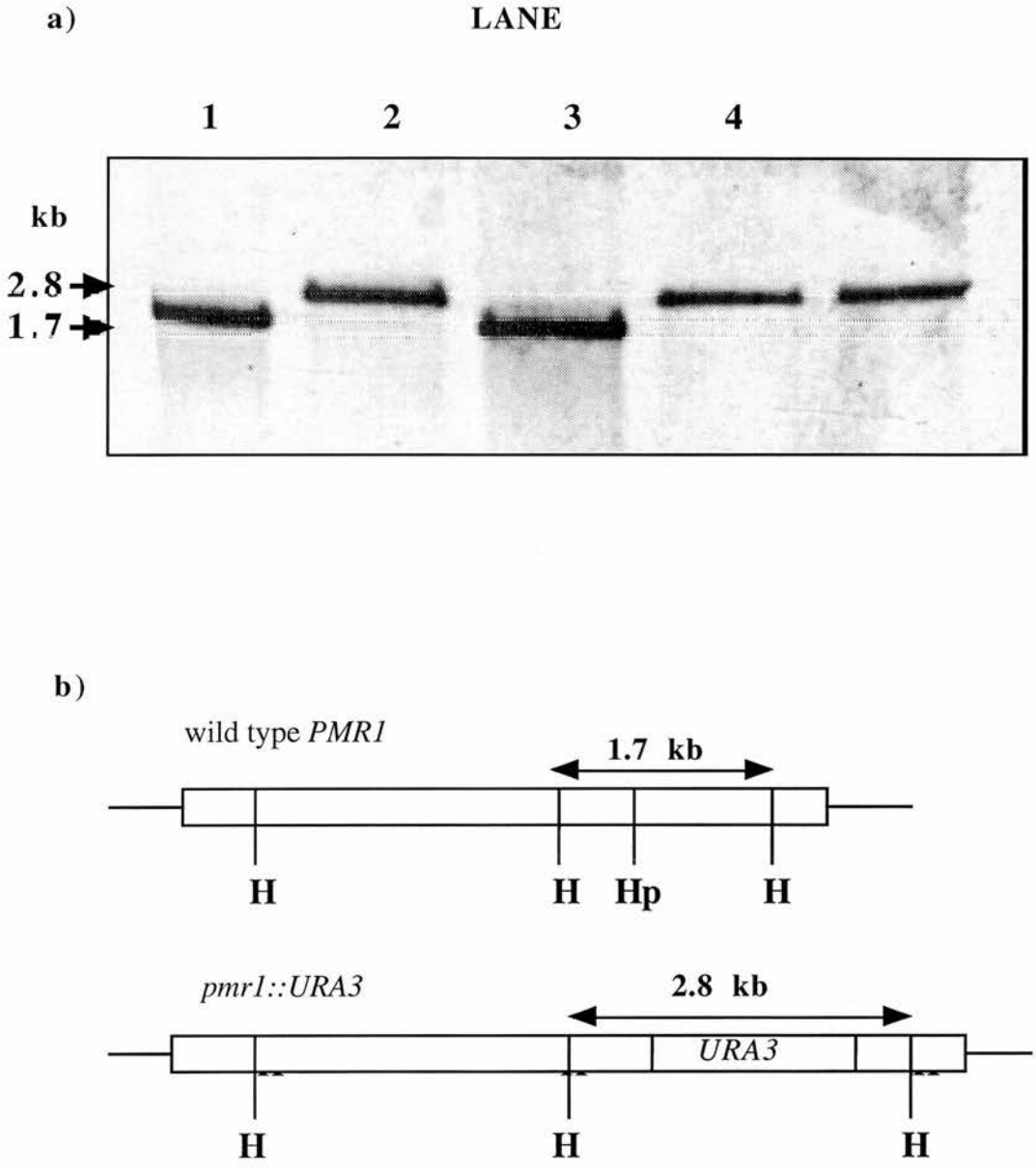
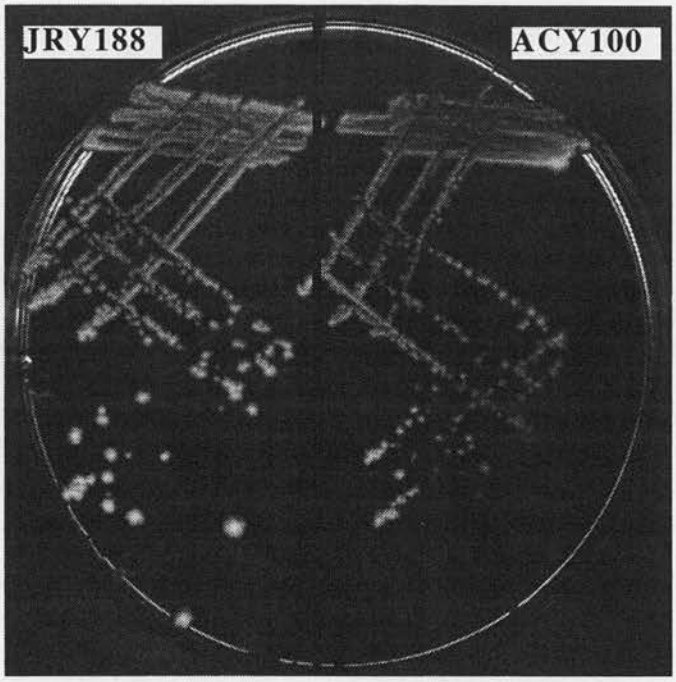


Figure 3-1 Southern blot analysis of potential *pmr1-2::URA3* disruptants.

a



b

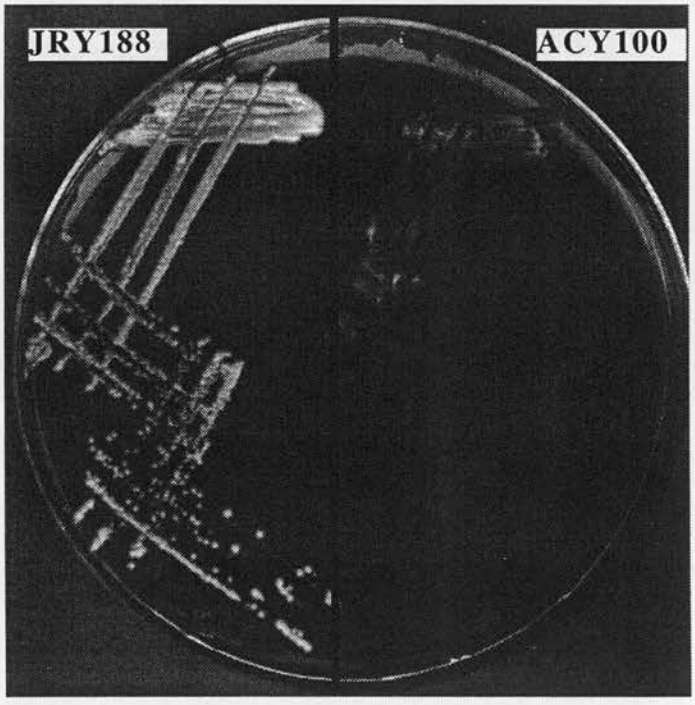


FIGURE 3-2 Strain ACY100 is sensitive to EGTA. The yeast strains JRY188 and ACY100 both grow normally on YPD (**a**). On YPD containing 25 mM EGTA both strains grow slower but ACY100 is more sensitive (**b**). Plates are shown after 3-4 days incubation at 24°C

FIGURE 3-3 A *pmr1* null mutant secretes a Kex2p processed β-lactamase. JRY188 and ACY100 were transformed with pYJS50. Ura+ transformants were selected for. Transformants were grown overnight in selective media and these precultures inoculated into 50 ml of YPD. YPD is a low Ca^{2+} medium (Antebi and Fink, 1992; Ohya *et al.*, 1984) and the *pmr1* null phenotype is displayed. After growing to an OD_{600} of about 2, cells were spun down for 5 min at 3000 g. 30 ml of supernatant from both cultures were subjected to TCA precipitation. All the precipitated material was loaded onto a 12% SDS-PAGE gel. The amount of protein loaded was not determined. **Lane 1**: contains protein A tagged recombinant proteins which act as molecular weight markers (Zeuco and Boyd 1992). **Lane 2**: material from JRY188 and **Lane 3**: material from the *pmr1* mutant. After electrophoresis the gel was western blotted. Primary antibody was rabbit anti-β-lactamase (1/1000 dilution) and the secondary antibody donkey-anti-rabbit IgG HRP conjugate (1/5000 dilution). Detection was by the ECL system (Amersham).

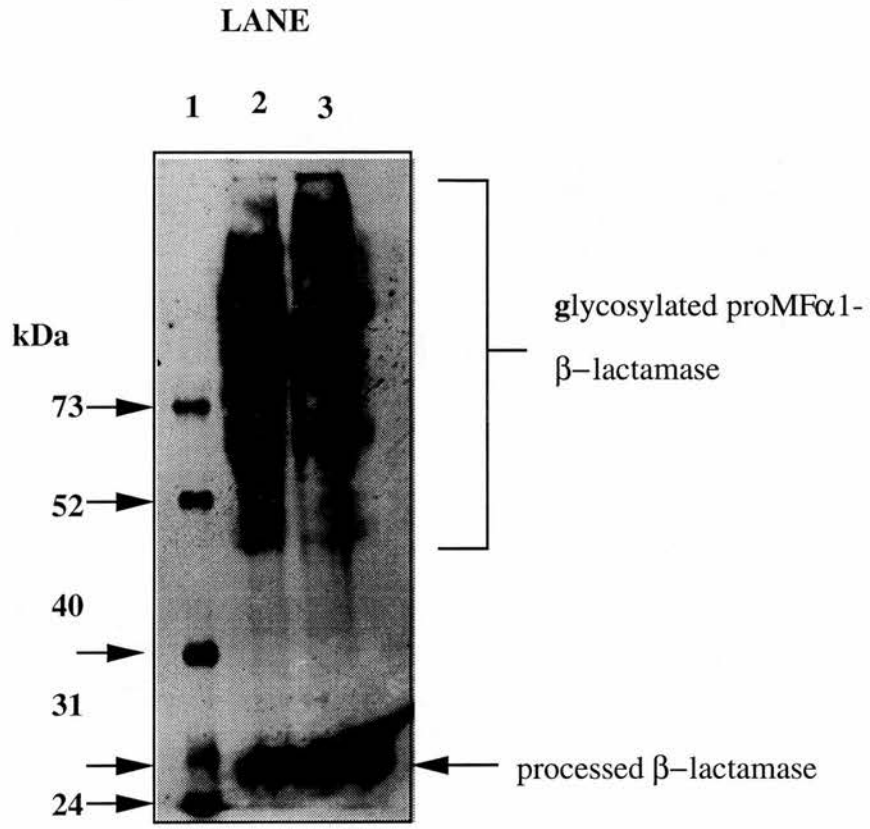


FIGURE 3-3 A *pmr1* null mutant secretes a Kex2p processed β-lactamase

demonstrating that the protein has probably been exposed to Kex2p and passed through the Golgi. This experiment demonstrates that proteins secreted by *pmr1* null mutants pass through a late Golgi compartment. It does not rule out the possibility of an earlier compartment being bypassed. There is also the possibility that protein trafficking is altered in *pmr1* null mutants. This could result in proteins being exposed to some Kex2p-like activity in a compartment not on the normal secretory pathway. To investigate this latter possibility a ACY100*kex2::LEU2* strain was made (data not shown). If proteins secreted by this strain were not processed then this would demonstrate that processing was Kex2p-dependent and prove transport through the late Golgi was indeed required for processing. In these experiments a different fusion protein was used to monitor transport and processing due to lack of anti-β-lactamase antibody. The plasmid YCpGALFSABL, which carries a streptavidin β-lactamase gene fusion with an α-factor signal sequence, was transformed into JRY188, a *kex2::LEU2* strain, a *pmr1::URA3* strain, and a *pmr1::URA3 kex2 ::LEU2* double mutant. Expression is under control of the *GAL1/10* promoter and the protein can be easily detected using biotin-HRP (Dr. Greg Stewart, personal communication). Despite several induction protocols the *pmr1::URA3 kex2::LEU2* double mutant failed to grow on galactose. The possibility that the streptavidin fusion protein was depleting the cell of biotin was a possibility but addition of 100 mM biotin to the media did not improve growth and this experiment was abandoned.

Antebi and Fink (1992) have since demonstrated that invertase secreted from *pmr1* strains receives mannose residues throuth α1,6 and α1,3 linkages. These are hallmarks of early and late Golgi processing steps respectively. The extent of outer chain processing was greatly reduced . They also showed that *pmr1* strains secrete a high molecular weight, highly glycosylated form of α-factor precursor and a little mature α-factor. This is a profile similar to that of invertase secreted by a *kex2*

mutant. Also secreted is a ladder of α-factor forms between 18 and 28 kDa which may represent early Golgi glycoslation and partial processing intermediates. Harmsen *et al* (1993) have also demonstrated that both Kex2p-processing and outer-chain glycosylation (at a reduced level) occurs in strains secreting the heterologous protein guar αGal under *MFα1* signal.

In conclusion these experiments demonstrate that a partial or total bypass of the Golgi apparatus does not explain the pleiotropic phenotype of *pmr1* mutants.

3-5 ACY100 has an altered Golgi organisation

Bryant and and Boyd (1993) developed an immunoaffinity procedure for the isolation of Kex2p-containing organelles. Affinity-purified anti-Kex2p antibodies were bound to fixed *S. aureus* cells (Pansorbin) forming an immunoadsorbant (ImAd). Using this ImAd they were able to isolate sealed organelles that were essentially free of contamination from other secretory organelles but enriched for the Golgi enzymes Kex2p, Kex1p, and dipeptidylaminopeptidase A (DPAP A). They proposed that they had isolated a single yeast Golgi compartment containing all three late processing enzymes. They also proposed that this compartment was likely to be the functional equivalent in yeast of the mammalian TGN. This procedure was used to isolate the Kex2p-containing organelles from *pmr1* mutants in low and high Ca^{2+} media as described below.

3-6 Affinity purification of the anti-C-terminal Kex2p antibodies

Affinity-purified anti-Kex2p antibodies were a generous gift from Dr. Nia Bryant. A Kex2p-βGal fusion protein was also provided to allow purification of more antibodies. This fusion protein was conjugated to Affi-gel (Bio-Rad) and packed in a column. Immune serum was applied and affinity-purified antibodies produced. It was

important to check that the affinity-purified anti-Kex2p antibodies recognised a single band on western blots corresponding to Kex2p. **FIG 3-4** shows that the purified antibodies recognise a single band of about 130 kDa in a *KEX2* strain. Kex2p has a predicted size of 90 kDa but has an apparent molecular weight of 135 kDa due to the high net negative charge that it carries in its C-terminal domain (Fuller *et al.* 1989). No band is detected in a *kex2* strain. (A partial N-terminal fragment of Kex2p could be synthesized in this strain. However in light of data shown in **FIG 3-5** if such a fragment exists it has no significant Kex2p activity). This demonstrates the antibodies used below are specific for Kex2p.

3-7 Comparison of Kex2p-containing membrane vesicles immunoisolated from a *pmr1* mutant and a *PMR1* strain grown in low Ca^{2+} media

Kex2p-containing vesicles were immunoisolated from a *PMR1* (JRY188) and a *pmr1* (ACY100) strain grown in YPD. The recovered material was assayed for various subcellular marker enzymes. Nearly 80% of Kex2p activity was recovered, along with 25% DPAP A activity, from a *PMR1* strain (**FIG 3-6**). These values are almost identical to those described by Bryant and Boyd (1993). Virtually no carboxypeptidase Y or dipeptidylaminopeptidase B (both vacuolar markers) were recovered. Similarly the level of the endoplasmic reticulum marker NADPH cytochrome c reductase was negligible. Furthermore the fraction is free of GDPase activity suggesting that the recovered material is free from other Golgi subcompartments. Material recovered from a *pmr1* strain differs in 2 ways: 1) DPAP A previously recovered in a *PMR1* strain is absent; 2) 55% of GDPase activity is now recovered (**FIG 3-6**).

Antebi and Fink (1992) failed to demonstrate any dramatic mislocalization of various

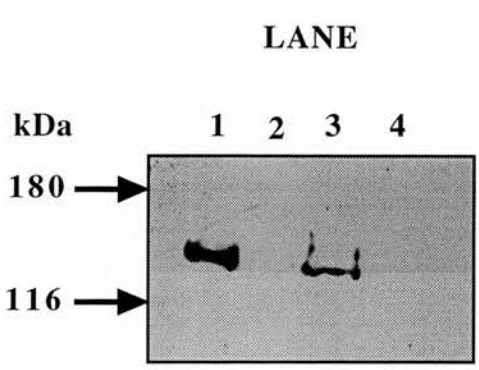


FIGURE 3-4 Affinity purified anti-Kex2p antibodies recognise a single band of the correct size in a *KEX2* strain. To demonstrate specificity of the affinity-purified antibodies yeast extracts were made from JRY188 and a JRY188*kex2::LEU2* mutant (ACY110). Samples of these extracts were run on a 12% SDS-PAGE gel and after electrophoresis western blotted. 20 μg of protein was loaded in each lane as determined by the BCA protein concentration determination assay kit (see Materials and Methods). Samples from JRY188 were loaded in lane **1** and **3**, The samples from ACY110 were loaded in lane **2** and **4**. Primary antibody was rabbit anti-Kex2p (1/1000 dilution) and the secondary antibody donkey-anti-rabbit IgG HRP conjugate (1/5000 dilution).

The *kex2* mutant strain ACY110 was made as follows. Plasmid pGA1070 (G. Ammerer) carries part of the *KEX2* gene which is disrupted by a functional *LEU2* gene. This plasmid was digested with *Bam*HI and *Sph*I to release a fragment containing the *kex2::LEU2* disruption. This fragment was gel purified and used to transform JRY188. Leu^+ colonies were selected and these transformants should have had their chromosomal copy of *KEX2* replaced with the *kex2::LEU2* disruption. Disruption of the functional *KEX2* gene was confirmed by growing transformants in selective media to an OD_{600} of 1 and assaying for Kex2p activity in yeast extracts prepared from the cultures (**figure 3-5**).

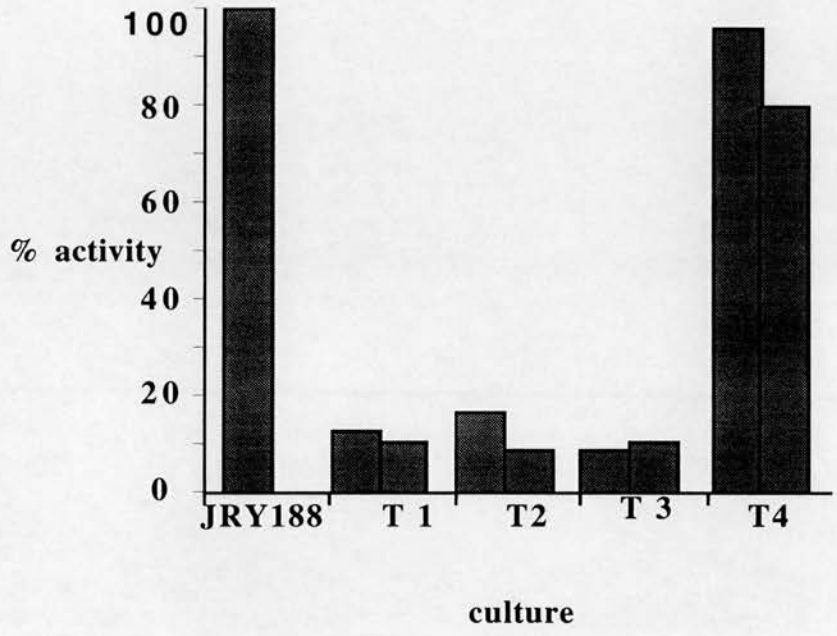


FIGURE 3-5 Confirmation of *kex2::LEU2* disruption. 10 ml Cultures of JRY188 and 4 possible disruptants were grown up in minimal media to an OD_{600} of about 1. Cells were spun down for 5 min at 3000 g. Whole-cell yeast extracts were produced by glass bead lysis. 50 µl samples were assayed for Kex2p activity (see materials and methods **2-20a**). T1-T3 are clearly disruptants having a 90% reduction in activity. T4 appears to have a functional *KEX2* gene and the disrupting DNA fragment has presumably integrated at the wrong locus. Activities are expressed as % of wild type activity and have been normalized for OD_{600}. Two assays were performed for each disruptant and both values are shown.

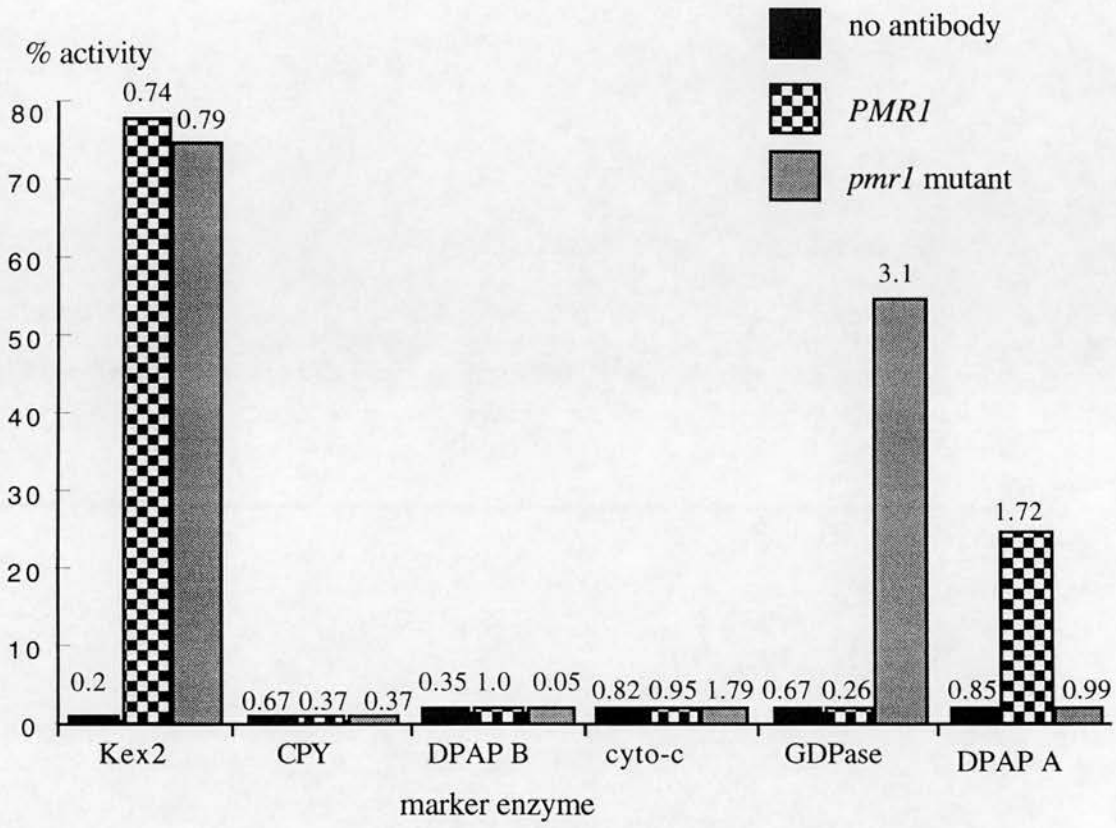


FIGURE 3-6 The profile of subcellular marker enzymes in Kex2p-containing vesicles immunoisolated from a *pmr1* mutant and a *PMR1* strain. 500 ml cultures of *pmr1* and *PMR1* strains were grown in YPD (a low Ca^{2+} medium) to an OD_{600} of about 1. Yeast cell homogenates were prepared from spheroplasts homogenized in a Dounce homogeniser (10-15 strokes). 30 OD_{600} equivalents of homogenate were incubated with 50 µl of immunoadsorbant (ImAd) for 2-3 hours in the cold room on a rotating wheel. As a control Pansorbin without anti-Kex2p antibodies bound was incubated with homogenate. Virtually no marker activity is recover by Pansorbin alone. After washes assays for each marker were carried out. The numbers above the bars are the standard deviations, the sample number was three. Activities are expressed as a % of the enzyme activity in the original homogenate.

marker enzymes in a *pmr1* mutant. They proposed that loss of Pmr1p from Golgi membranes alters the Ca^{2+} level of either the Golgi lumen or the cytoplasm or both. This could result in activities of several Golgi enzymes being altered. Both Kex2p and GDPase require Ca^{2+} for their activity and this may be reduced in a *pmr1* mutant. In contrast to Antebi and Fink's (1992) work the results summarised in **FIG 3-6** suggest that loss of Pmr1p results in a breakdown in normal Golgi subcompartmentalization. As a result various processing enzymes may now find themselves in sub-optimal environments and have reduced activity. Whether this breakdown is due to the failure of proper retention mechanisms or of various Golgi membranes fusing is unclear. The supersecreting phenotype of *pmr1* mutants could then be explained as follows. If various Golgi compartments fuse and form a single compartment there are now fewer steps for heterologous proteins to pass through. This might result in a rate-limiting transport or modification step being eliminated.

Another explanation for this supersecreting phenotype could be that altered Ca^{2+} levels affect the interaction of Kar2p with malfolded proteins in the endoplasmic reticulum. Suzuki *et al.*(1991) have shown that in CHO and COS cell lines the chelation of Ca^{2+} in whole-cell extracts by EGTA results in dissociation of BiP (the Kar2p homologue) from T-cell receptor a chain (TCR) variants. This release was dependant on Mg^{2+} and ATP and addition of Ca^{2+} was found to stabilize the association. Supporting this it has been shown that Ca^{2+} inhibits the ATPase activity of BiP (Kassenbrock and Kelly 1989). Suzuki *et al* (1991) went on to demonstrate that treatment of these cell lines with the Ca^{2+} ionophore A23187 results in the secretion or cell surface expression of the soluble- and membrane-associated variants of TCR respectively. A specific inhibitor of the endoplasmic reticulum Ca^{2+}ATPase had the same effect. BiP was not secreted. Antebi and Fink (1992) have shown that Kar2p is not secreted nor relocalized in a *pmr1* mutant. A supersecreting phenotype

could result if malfolded heterologous proteins previously retained in the endoplasmic reticulum, by Kar2p, are released as a result of the *pmr1* mutation altering Ca^{2+} levels.

3-8 High Ca^{2+} levels do not reverse the changes in Golgi organisation in a *pmr1* null mutant

Kex2p-containing vesicles were immunoisolated from a *pmr1* mutant grown in high and low Ca^{2+}. **FIG 3-7** shows that high Ca^{2+} concentration has no effect on the profile of recovered material. So far all the observed phenotypes of *pmr1* null mutants have been reversible by adding Ca^{2+} into the growth media. Although the profiles of recovered material were not reversed an improvement in growth rate was observed suggesting that Ca^{2+} had suppressed the *pmr1* phenotype. These results suggest that the changes in Golgi organisation are not reversed and therefore the high Ca^{2+} levels act in some other way. It has been proposed by Antebi and Fink (1992) that the presence of extracellular Ca^{2+} drives more Ca^{2+} into the Golgi compartments thereby restoring Ca^{2+}-dependent events. Another possibility is that the activity of some other protein is able to compensate for the loss of the Pmr1p in the presence of high Ca^{2+} levels. Okorokov *et al.* (1993) have demonstrated biochemically the presence of another Ca^{2+}ATPase in yeast Golgi. Perhaps high Ca^{2+} levels are able to increase the activity of this pump or increase its level of expression.

3-9 Summary

1) Proteins secreted by *pmr1* null mutants pass through the Golgi apparatus. This was demonstrated by the Kex2p processing of a α-mating factor β-lactamase fusion protein. This is supported by Antebi and Fink (1992) and Harmsen *et al.*, (1993).

2) The localization of proteins in the Golgi is altered in a *pmr1* mutant grown in low Ca^{2+} media. In particular GDPase now colocalises with Kex2p, and DPAP A is absent from the Kex2p-containing compartment.

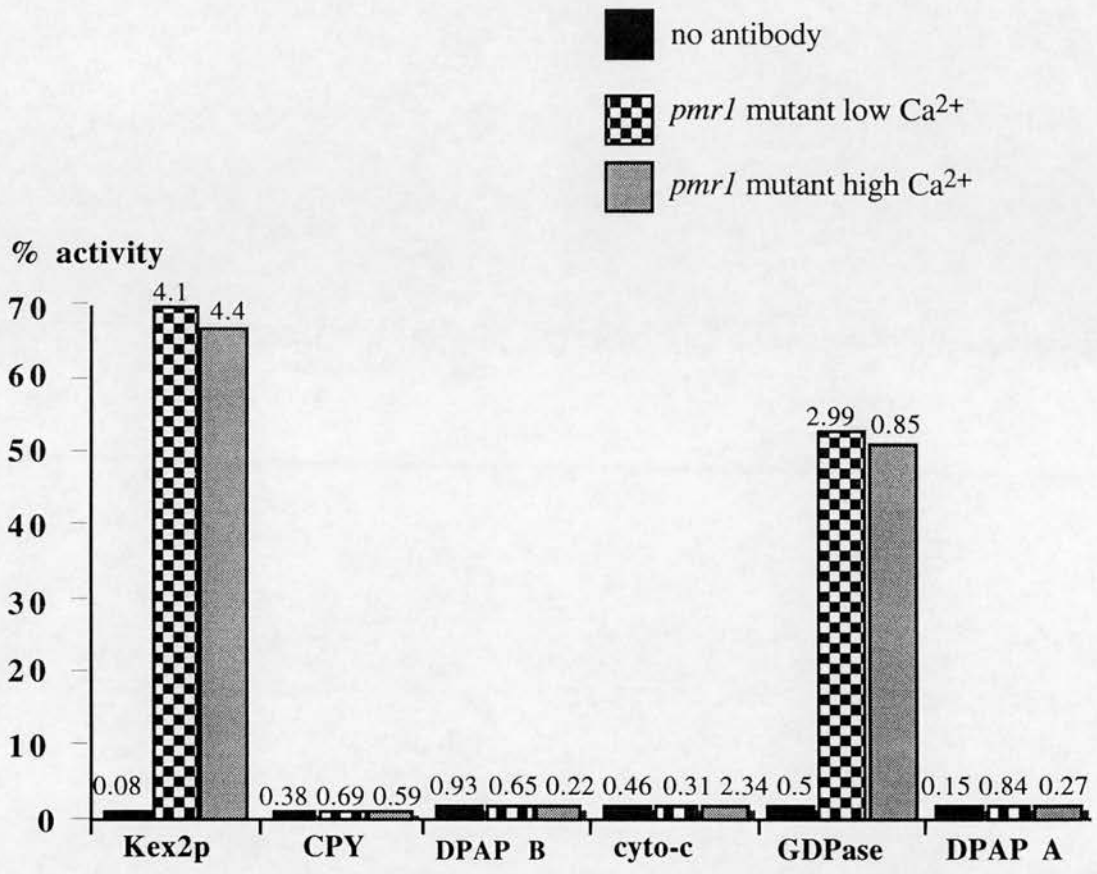


FIGURE 3-7 High Ca^{2+} levels do not reverse the changes in Golgi organisation in a *pmr1* null mutant. 500 ml cultures of the *pmr1* mutant were grown to an OD_{600} of 1 in low (YPD) and high Ca^{2+} (YPD+ 10 mM $CaCl_2$) media. Kex2p-containing vesicles were immunoisolated using ImAd as described in **figure 3-6**. Recovery of Kex2p activity was less compared with **figure 3-6**. This is most likely to be a result of using a different batch of affinity-purified antibodies. Activities are expressed as a % of the enzyme activity in the original homogenate. The numbers above the bars are standard deviations, the sample number was three for each assay.

3) These changes in Golgi organisation do not appear to be reversed by addition of Ca^{2+} to the media. This is somewhat surprising since all the phenotypes of *pmr1* mutants so far reported are reversible by the addition of 10 mM $CaCl_2$ into the growth medium.

pmr1 mutants have altered Golgi structure and function. In particular the subcellular localization of Kex2p, DPAP A, and GDPase appears to be significantly altered.

In conclusion the evidence presented in this chapter supports the idea that Ca^{2+} homeostasis in *S. cerevisiae* is important in maintaining proper Golgi apparatus structure and function.

Chapter 4
Isolation and characterization of the compartment containing a protein A tagged Pmr1p protein

4-1 Introduction

Antebi and Fink (1992) demonstrated that Pmr1p localizes to a "Golgi-like organelle" by subcellular fractionation and indirect immunofluorescence. Subcellular fractionation shows that membranes containing Pmr1p fractionate away from endoplasmic reticulum, plasma membrane, and vacuole. Pmr1p largely comigrates on sucrose gradients with the Golgi markers GDPase and Kex2p (Antebi and Fink, 1992). However the distribution of Pmr1p does not strictly follow the profile of these markers suggesting a different distribution within the Golgi. Indirect immunofluorescence shows that Pmr1p distribution has a punctate staining pattern. In *S. cerevisiae* this pattern is consistent with Golgi distribution (Redding *et al.*,1991; Franzusoff *et al.*, 1991; Cleaves *et al.*, 1991; Roberts *et al.*, 1992; Cooper and Bussey, 1992.). The Pmr1p pattern overlaps Kex2p and Sec7p with 26% and 17% colocalization respectively. Kex2p and Sec7p colocalize with a higher frequecy (58%). This is thought to be because they occupy different profiles of Golgi compartments. It seems likely that Pmr1p occupies a different profile of Golgi compartments compared with known Golgi markers. A better understanding of this distribution could help in understanding the function of Pmr1p and the nature of the null phenotype. This chapter describes experiments designed to isolate the Pmr1p-containing organelle(s) from yeast and to characterize them.

4-2 Construction of a gene encoding a Pmr1p-protein A fusion protein

The *PMR1* gene was tagged with part of the *S. aureus* protein A gene encoding the IgG F_C binding domain with the aim of isolating vesicles containing the Pmr1p-protein-A fusion protein using IgG-Sepharose (Nilsson *et al.*, 1985). This circumvents the need to raise and affinity purify polyclonal antibodies. A 394 bp C-terminal fragment of the *PMR1* gene was amplified by PCR. The 5' primer (E964) anneals within the *PMR1* gene at an *Sst*II site (underlined below). The 3' primer

(F023) removes the stop codon from the *PMR1* gene and introduces a *Sal*I site (underlined below) so that the protein A DNA can be fused in frame.

Primer E964 : 5' TCC CCG CGG CCT GTA TCA TCG T 3'

Primer F023 : 5' GCG GTC GAC CCA ACA TTT GAG AAA TAC GTT GAG TC 3'

PCR conditions were 1 min 95°C, 1 min 55°C, 1min 72°C for 20 cycles using Vent polymerase. This PCR product was cloned as an *Sst*II/*Sal*I fragment along with a *Sal*I/*Hin*dIII protein A fragment into pBluescript II KS$^-$ previously digested with *Sst*II and *Hin*dIII. The protein A DNA fragment source was pKpraSH (Bryant, 1992) and is 0.5 kb. This plasmid was called pKSPMR1prA.

The 5' portion of the *PMR1* gene including its own promoter was introduced into YCplac22 (Geitz and Sugino, 1988) as a *Pvu*II/ *Sst*II fragment along with an *Sst*II/ *Hin*dIII fragment containing the 3' portion fused to protein A sequence from pKSPMR1prA. YCplac22 had been digested with *Sma*I and *Hin*dIII. The result is a centromeric plasmid carrying the *PMR1* gene which has the DNA encoding the F_C binding domain of protein A fused to its 3' end. This plasmid is called pSpaM (see **figure 4-1**). The nucleotide sequence of the 394 bp *PMR1* PCR product was determined by DNA sequencing to check for PCR errors. None were detected.

4-3 pSpaM complements a *pmr1-2::URA3* mutation and a fusion protein of the correct size is detectable in transformants

Strain ACY100 was transformed with pSpaM. ACY100 transformants harbouring pSpaM gained the ability to grow on low Ca^{2+} media containing 25 mM EGTA (**FIG 4-2**). This suggests that SpaM has some activity equivalent to untagged Pmr1p, perhaps indicating a normal localization. A fusion protein of the predicted

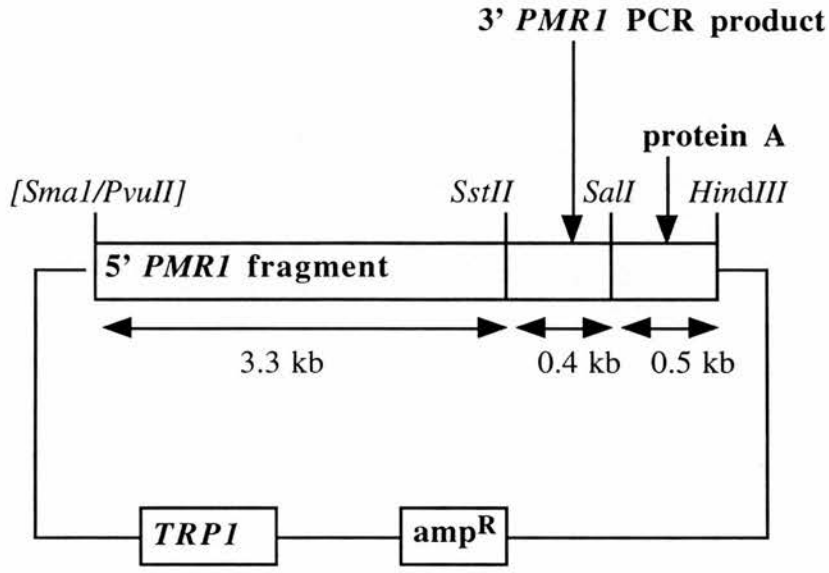


Figure 4-1 Schematic of pSpaM. Plasmid pSpaM was constructed using a YCplac22 backbone; the *TRP1* and amp^R selectable markers are shown. The relative sizes of the 5' portion of the *PMR1* gene and the 3' PCR fragment are shown above. The actual sizes of the 2 fragments are 3325 bp and 394 bp respectivley.

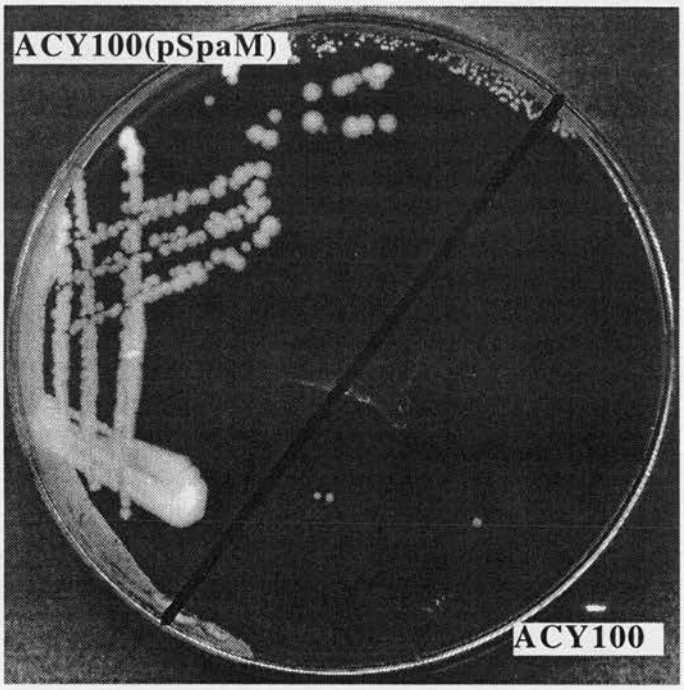


FIGURE 4-2 pSpaM complements a *pmr1* mutation. ACY100 and ACY100(pSpaM) were plated on YPD containing 25 mM EGTA and incubated at 24°C for several days. ACY100 has only formed a few single colonies. ACY100 grows well when transformed with pSpaM.

molecular weight (around 116 kDa) was detected in JRY188(pSpaM) transformants (**FIG 4-3** lanes **1** and **3**).

4-4 SpaM fusion protein can be recovered using IgG-Sepharose

IgG-Sepharose was used to recover membranes containing the SpaM fusion protein from JRY188(pSpaM) transformants. **Figure 4-4** shows a western blot demonstrating recovery of the fusion protein from transformants. Heavily stained diffuse bands are seen in the IgG-Sepharose lane (lane **1**), the JRY188 control lanes (lanes **2** and **3**), and the lanes containing immunoisolated material (lanes **4** to **7**). These bands are thought to be complexes of IgG heavy and light chains released from the IgG-Sepharose by boiling of the sample (indicated on **figure 4-4**). Monomeric heavy and light chains caused a problem with the ECL detection by producing a very intense signal which greatly reduced the sensitivity of detection of SpaM. This was overcome by removing the bottom part of the nitrocellulose filter, hence the straight line at the bottom of **figure 4-4** Two distinct bands are seen in lanes **4** to **7**. These are in the correct size range for the SpaM fusion protein. The lower may be a degradation product.

4-5 Characterization of the material immunoisolated using IgG-Sepharose

Material recovered using IgG-Sepharose from the pSpaM transformants contains significant amounts of vacuolar marker enzymes (**figure 4-5**). 27.5% CPY (a soluble vacuolar marker) activity is recovered as well as 25% of DPAP B activity (a vacuolar membrane marker). A small amount of the Kex2p and DPAP A activities are also recovered but negligble NADPH cytochrome-c reductase and GDPase activity is detected. This data suggests that the SpaM fusion protein has a predominately vacuolar localization.

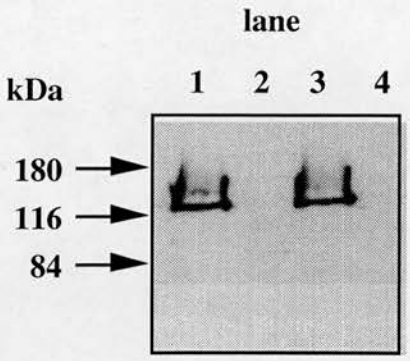


Figure 4-3 JRY188(pSpaM) transformants contain Pmr1p-protein A fusion protein of the correct molecular weight. JRY188 was transformed with pSpaM. 50 ml cultures of JRY188(pSpaM) and JRY188 were grown in minimal media to an OD_{600} of about 1. Cells were harvested by centrifugation (3000 g 5 min) and glass bead extracts produced. 25mg of protein was loaded in each lane and run on a 12% SDS-PAGE gel. The gel was western blotted to nitrocellulose. The fusion protein was detected with a rabbit anti-bovine IgG-HRP conjugate using the ECL detection protocol. **Lanes 1** and **3** contain material from pSpaM transformants. **Lanes 2** and **4** contain material from untransformed JRY188.

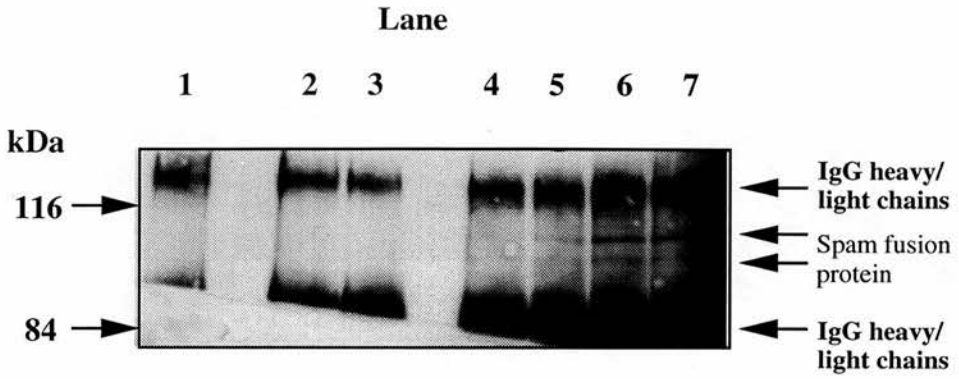


Figure 4-4 Recovery of vesicles containing the SpaM fusion protein. 100 ml cultures of JRY188 and of 4 JRY188(pSpaM) transformants were grown to an OD_{600} of about 1. The cells were spun down, spheroplasted, and the homogenised in a Dounce homogenizer. The equivalent of 30 OD_{600} units of homogenate from each culture was incubated with IgG-Sepharose. Following washing the IgG-Sepharose was resuspended in SDS-PAGE sample buffer and incubated at 70°C for 5 minutes. The IgG-Sepharose was then allowed to settle out by gravity and the supernatent was loaded onto a 10% SDS-PAGE gel and after electophoresis western blotted. Lane **1**: supernatent from IgG-Sepharose alone. Lanes**2** and **3**: supernatent from IgG-Sepharose incubated with homogenate from JRY188. Lanes**4** to **7**: supernatent from homogenate from JRY188(pSpaM) transformants. The blot was probed with a rabbit anti-bovine IgG-HRP conjugate and developed using the ECL protocol.

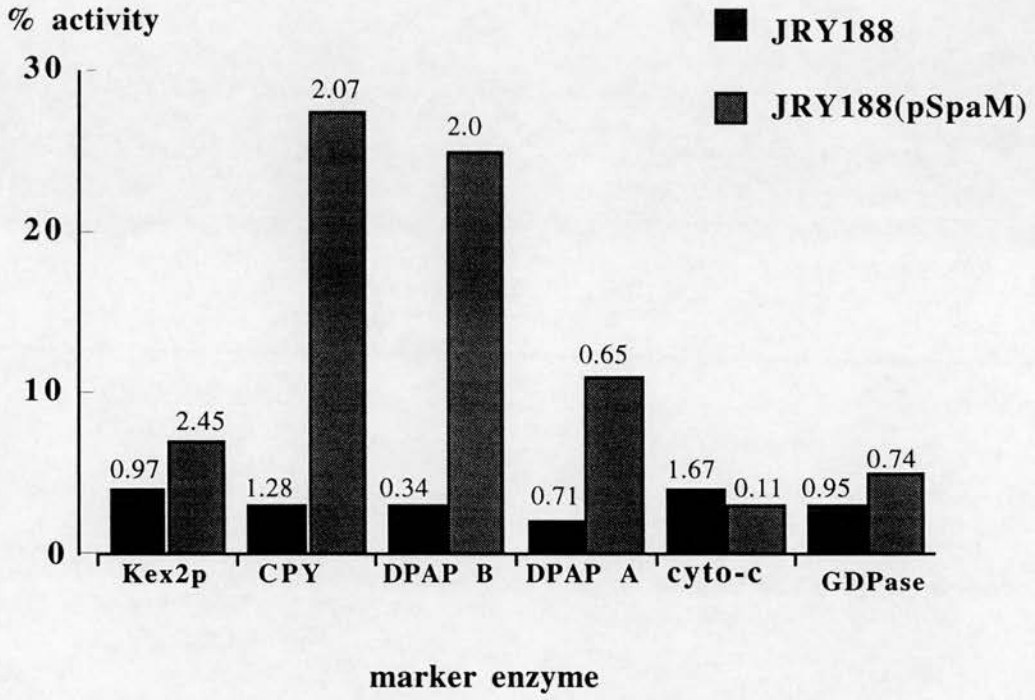


Figure 4-5 Profile of subcellular markers recovered from JRY188 and JRY188(pSpaM) using IgG-Sepharose.

500 ml cultures of JRY188 and JRY188(pSpaM) were grown in minimal selective media overnight to an OD_{600} of about 1. Cells were spun down and homogenates produced. 30 OD_{600} equivalents of homogenate were incubated with 50µl of IgG-Sepharose which had previously been blocked and equilibrated as described in Material and Methods. After incubation for several hours at 4°C followed by washes the immunoisolated material was assayed for various subcellular marker enzymes. Assays were carried out in triplicate (3 lots of 50µl IgG-Sepharose) for each marker. Activity is expressed as a % of the total activity in the homogenate and the numbers above each bar are standard deviations.

Figure 4-6 Subcellular fractionation supports a vacuolar distribution. A 500 ml culture of JRY188(pSpaM) was grown in minimal selctive media overnight to an OD_{600} of about 1. Cells were spun down, spheroplasted, and homogenised. After a clearing spin the homogenate was loaded onto an 18%-54% (w/w) sucrose gradient. After spinning for 2.5-h at 174 000 g 1 ml fractions were taken. 600 µl of each fraction was diluted 2 fold with 10 mM HEPES pH 7.5 and subjected to TCA precipitation. The rest of the fraction was stored at -20°C until required for enzyme assays. After precipitation the sample was spun for 10 min at 10 000 g and a sticky white pellet formed. Due to sucrose precipitating this pellet often proved difficult to resuspend. The pellet was resuspended in SDS-PAGE sample buffer heated for several minutes at least (up to 20 min to dissolve the sucrose) and loaded onto a 10% SDS-PAGE gel. After electrophoresis western blotting was performed and a rabbit anti-bovine IgG-HRP conjugate used to detect the SpaM fusion protein. ECL detection protocol was used. CPY (a vacuolar marker), GDPase, and Kex2p (both Golgi markers) activities were assayed in each fraction. Assays were carried out as described. 50 µl aliquots from each fraction were used in the enzyme assays. Activity units are arbitrary.

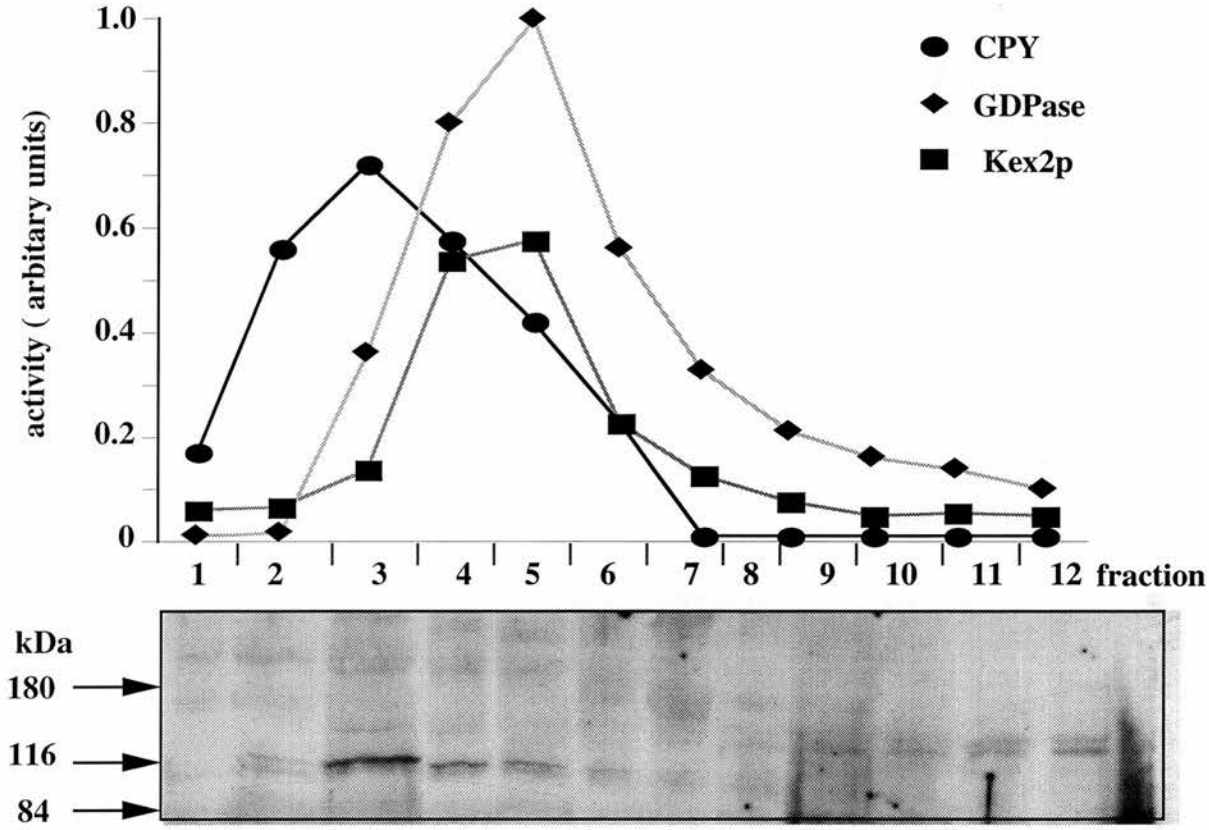


Figure 4-6 Subcellular fractionation supports a vacuolar distribution

4-6 Subcellular fractionation supports a predominantly vacuolar distribution

Homogenate from JRY188(pSpaM) was separated on an 18%-54% (w/w) sucrose gradient (Antebi and Fink, 1992). The distribution of the SpaM fusion protein was investigated by western blotting of fractions from this gradient. **Figure 4-6** shows that the majority of the SpaM protein was found in fraction **3** which corresponds to the peak of CPY activity (a vacuolar marker).

Clearly addition of the protein A sequence to the C-terminus of Pmr1p has affected its subcellular distribution. Mutagenesis studies on Kex2p, Kex1p, and DPAP A (Wilcox *et al.*, 1992; Cooper and Bussey, 1992; Roberts *et al.*, 1992; Nothwehr *et al.*, 1993) have shown that removal of the proteins' cytoplasmic tails eliminates retention in the Golgi complex and that the mutant proteins are delivered to the vacuole. An 8 amino acid region of DPAP A (Ste13p) is the most important region for its retention (Nothwehr *et al.*, 1993). Detailed mutational analysis showed a Phe-X-Phe-X-Asp motif was absolutely required for efficient retention. My analysis of the predicted amino acid sequence of Pmr1p (Rudolph *et al.*, 1989) shows that this motif is present in the C-terminus region between residues 852 and 856 (this is perhaps further evidence for a Golgi localizaton for Pmr1p). A possible explanation for SpaM's vacuolar localization is that addition of protein A in someway interferes with the interaction between this or some other retention motif and the corresponding receptor molecule. This interference results in Pmr1p's delivery to the vacuole. Interference could simply be a steric one or could be due to misfolding of the tagged protein. It has been shown that addition of protein A to the C-terminus of Kex2p also results in it's delivery to the vacuole (Bryant and Boyd, in press). Both SpaM and protein A tagged Kex2p complement some aspects of the relevant mutations. This suggests that enough mutant protein is present in the Golgi at any one time to carry out

normal activity.

The experiments so far described in this chapter were carried out in JRY188 rather than ACY100. This was because of worries about aberrant protein trafficking and retention in a *pmr1* null mutant (see chapter 3 for example). It could be that in a cell expressing wild type as well as protein A-tagged Pmr1p that a competition for the retention motif receptor molecule occurs. It could be imagined that wild type Pmr1p would have a greater affinity for the receptor and be preferentially retained. The tagged Pmr1p would therefore be more likely to be found in the vacuole. To check this the IgG-Sepharose immunoisolation experiment was repeated in a *pmr1* null mutant. The profile of recoverd marker enzymes was however essentially the same (**figure 4-7**).

4-7 Tagging of Pmr1p with Strep tag

As well as tagging Pmr1p with protein A an attempt was made to tag Pmr1p with a 10 amino acid peptide sequence called Strep tag (Schmidit and Skerra, 1993). This sequence has a high affinity for streptavidin and allows a single-step purification of proteins using a suitable streptavidin affinity column. It was hoped that vesicles containing Strep-tagged Pmr1p could be isolated using magnetic beads coated with streptavidin. The tag also allows detection of proteins with streptavidin-HRP. Tagging with a short peptide sequence was performed because of worries about the effects of the large protein A tag on Pmr1p.

A similar strategy to that used to add protein A sequence to *PMR1* was used. First the 3' portion of the gene was amplified again using primer E964 (see above for sequence) as the 5' primer. The 3' primer sequence (18/94) encodes the Strep tag:

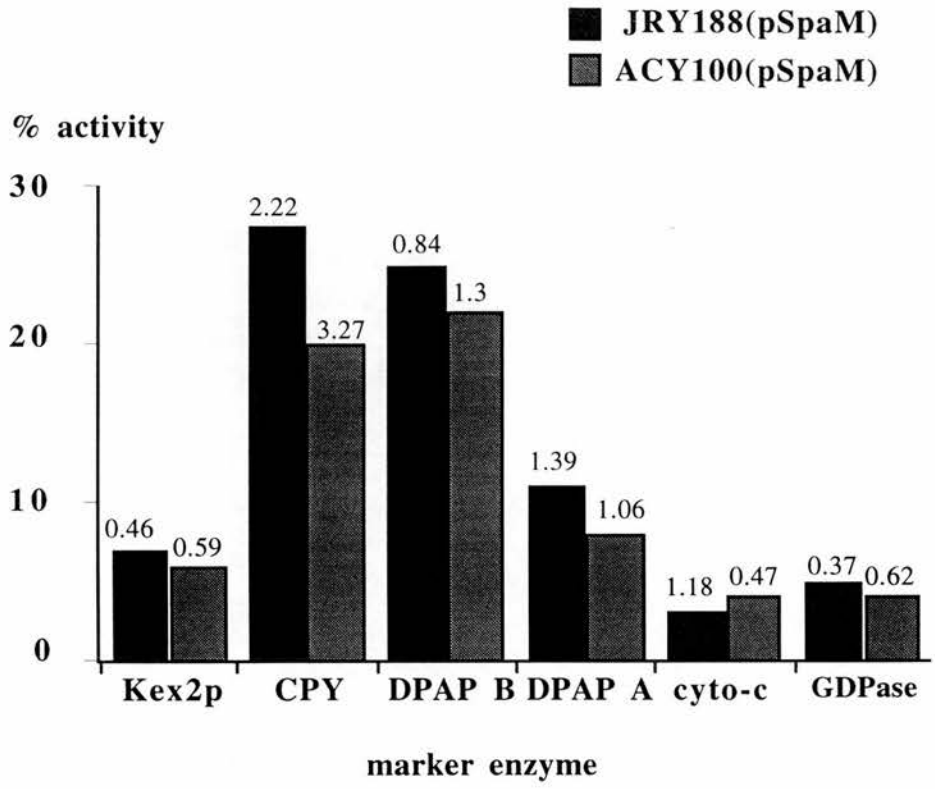


Figure 4-7. The profile of subcellular markers recovered from JRY188(pSpaM) and ACY100(pSpaM). Experimental procedures were as described in **figure legend 4-5**. A control culture of untransformed JRY188 was also used and negligible levels of all marker were recovered as before (data not shown). Activities are a % of the total activity in the homogenate. The numbers above the bars are the standard deviations (sample number 3)

5' CCC GGG TTA ACC ACC GAA CTG CGG GTG ACG CCA AGC GCT AAC ATT TGA GAA ATA CGT TGA GTC TTC 3'

PCR conditions were 95°C 1 min, 55°C 1 min, 72°C 1 min, for 20 cycles. Vent polymerase was used. The 0.4 kb PCR product was cloned as a blunt product into the *Sma*I site of YEplac112 and YCplac22 (Geitz and Siguino, 1988). Clones were screened to isolate those with the *Sst*II site of primer E964 closest to the *Sst*I site of the polylinker. This allowed the 5' portion of the *PMR1* gene to be introduced as a 3.4 kb *Sst*I/*Sst*II fragment isolated from pSpaM. These two plasmids were called pYCpPMRStrep and pYEpPMRStrep.

4-8 Expression of Strep tagged Pmr1p

JRY188 was transformed with either pYCpPMRStrep or pYEpPMRStrep. No band could be detected in yeast extracts by western blotting (**figure 4-8**). A band of around 105 kDa was expected but no band in this region which was not seen in the control extract could be seen. The multiple bands seen in both the extracts made from transformed and untransformed culture are presumably endogenous proteins which contain biotin and are detected with the streptavidin-HRP. These proteins are likely to be carboxylases such as acetyl-CoA carboxylase, pyruvate carboxylase and urea carboxylase, all of which contain biotin as a cofactor. Several explanations for not detecting the tagged protein can be imagined such as a PCR error or a mistake in the primer sequence (at the synthesis level) or that the protein is degraded rapidly. Time considerations meant this line of investigation was not continued.

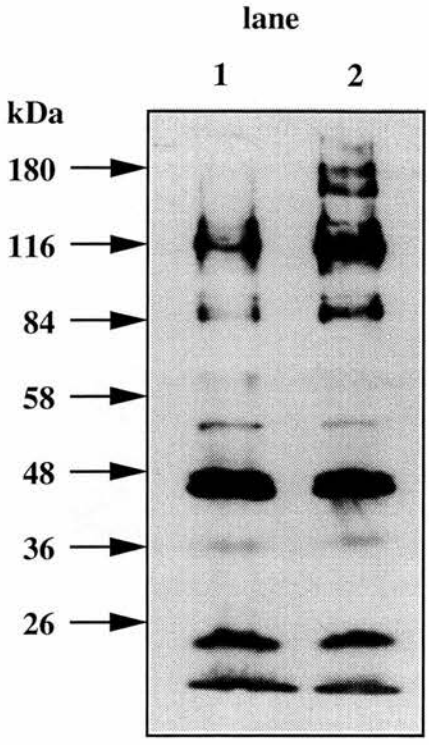


Figure 4-8 Attempt to detect a Strep tagged Pmr1p.

Several attempts were made to detect Strep tagged Pmr1p and the figure above shows a typical result. 100 ml cultures of JRY188 and JRY188(pYEpPMRStrep) were grown to an OD_{600} of about 1 in selective minimal media. Whole cell yeast extracts were produced from each culture by glass bead lysis. Extracts were analysed on a 10% SDS-PAGE gel and the gel was western blotted to nitrocellulose (30µg protein loaded per lane). A streptavidin-HRP conjugate was used in attempts to detect the tagged protein. The ECL detection system was used. **Lane 1**: extract from JRY188(pYEpPMRStrep). **Lane 2**: extract from JRY188.

4-9 Summary

1) A protein A tagged Pmr1p (SpaM) complemented the growth defect of ACY100 on low Ca^{2+} media containing EGTA initially suggestingthat the tagged protein had a correct subcellular localisation.

2) Characterization of the contents of isolated vesicles containing SpaM shows significant enrichment of the vacuolar markers CPY(27.5%) and DPAP B (25%).

3) Subcellular fractionation by separation on a sucrose gradient confirms a vacuolar localisation suggesting that the protein A tag has altered retention or targetting of Pmr1p.

4) An attempt to tag Pmr1p with the 10 amino acid Strep tag failed.

This work demonstrates that addition of a protein A tag to the C-terminal end of Pmr1p disrupts the retention mechanism in the Golgi for this protein in some way. The result was transport to the vacuole supporting the vacuolar default model for membrane proteins in *S. cerevisiae*.

Chapter 5
Localization of a vacuolar targeted protein tagged with a plasma membrane protein

5-1 Introduction

There is increasing evidence to suggest that the default pathway for membrane proteins in yeast leads to the vacuole (Roberts *et al.*, 1992; Cooper and Bussey, 1992; Wilcox *et al.*, 1992; Nothwehr *et al.*, 1993). This is in contrast to higher eukaryotic cells which have a default pathway ending at the plasma membrane. One prediction of the vacuolar default pathway model is that plasma membrane proteins should have a targeting signal. Plasma membrane targeting signals have already been identified in higher eukaryotes. Although the default pathway leads to the plasma membrane, signals which target proteins to specific areas of the plasma membrane do exist. These are of particular importance in polarised cells such as epithelial cells.

In chapter 4 the localization of a protein A-tagged Golgi protein, Pmr1p, to the vacuole was described. Bryant and Boyd (1995) have shown that Kex2p, a late Golgi processing enzyme, is also found in the vacuole when tagged at its C-terminus with protein A. These results suggest that normal retention of these proteins in the Golgi has been interfered with. One explanation for their vacuolar localization would be if they had entered a default pathway for membrane proteins which leads to the vacuole.

In this chapter experiments to identify a plasma membrane targeting signal are described. The general approach was to construct a Kex2p-protein A tagged protein to which a plasma membrane protein had also been added. The Kex2p-protein A fusion protein (SpaK) is known to localize to the vacuole (Bryant and Boyd, in press, Yeast). The entire sequence of Pma1p, a plasma membrane H^+-ATPase (Serrano *et al.*, 1986; Serrano, 1991), was added to SpaK. If this sequence contains a plasma membrane targeting signal, such a hybrid protein should be targeted to the plasma membrane and not to the vacuole.

5-2 Construction of a *KEX2-spa-PMA1* gene fusion

The *KEX2* gene, including the promoter was isolated from plasmid pNB64 (provided by Dr. N. Bryant) as a 3.6 kb *Kpn*I/*Bam*HI fragment. This was cloned into YCplac22 (Geitz and Sugino, 1988), previously digested with *Kpn*I and *Bam*HI, to produce plasmid YCplac22*KEX2*. A protein A fragment was amplified by PCR using primers 1793/93 and 1793/94 (sequences shown below). These primers were designed to introduce a *Bam*HI at the 5' end and a *Sph*I site at the 3' end (sites underlined).

1793/93 5' GGA TCC TAG CTT AAA AGA TGA CCC AAG C 3'

1794/93 5' GCA TGC AAT TCT TTA TCG TCA TCT TTT GGT GCT T 3'

The template DNA was plasmid pKprA (ref). PCR conditions were 95°C 1 min, 55°C 1 min, 72°C 1 min, for 20 cycles and Vent polymerase was used. This PCR product was cloned into the *Sma*I site of plasmid pK19 as a blunt fragment. The entire coding sequence of *PMA1* was amplified by PCR using genomic DNA from JRY188 as template. The primers used were designed to introduce *Sph*I sites (underlined) at both ends of the amplified fragment:

1791/93 5' GCA TGC ATG ACT GAT ACA TCA TCC TCT TC 3'

1792/93 5' GCA TGC TTA GGT TTC CTT TTC GTG TTG AGT 3'

The 2.8 kb fragment produced was cloned as a blunt fragment into the *Sma*I site of plasmid pK19. The first 100 bp of each end were checked by DNA sequencing to make sure the *PMA1* gene had been amplified. The sequence was confirmed and no PCR errors detected. The gene fusion was assembled by digesting plasmid YCplac22*KEX2* with *Bam*HI and *Sph*I and ligating in the *Bam*HI/*Sph*I protein-A fragment and the *Sph*I *PMA1* fragment simultaneously. This plasmid were called pSpaKP.

5-3 Detection of the tribrid fusion protein, expressed from pSpaKP, in yeast extracts

A fusion protein of 207 kDa was predicted from the amino acid sequence of the tribrid protein. **Figure 5-1** shows schematic drawings of the tribrid protein and of SpaK. **Figure 5-2** shows that a protein is detected, by virtue of the protein A tag, in a membrane fraction from whole-cell yeast extracts in this molecular weight range and that no equivalent band is seen in an untransformed strain. Rather than a distinct band a smear in the lane is observed. This is characteristic of a protein which has been heavily glycosylated *eg* invertase, when it is run under similar conditions (Orlean *et al.*, 1991)

The tribrid protein has Kex2p activity (**figure 5-3**) and complements the cold-sensitive growth phenotype of a *kex2* mutant (**figure 5-4**). However a Kex2p-protein A (SpaK) construct also complemented the cold-sensitive phenotype even though it localizes to the vacuole. This gave no clue to the subcellular localisation of the Pma1p-tagged Kex2p-protein A. Presumably enough of the respective fusion proteins are present in the Golgi at any one time to carry out the function(s) which prevent cold sensitivity.

5-4 Subcellular localization of the tribrid protein

The Kex2p activity of the tribrid protein allowed its subcellular localization to be determined. Yeast homogenates were separated by centrifugation through a sucrose gradient and **figure 5-5** shows that the Kex2p activity of the tribrid protein is found in the same fractions as wild type Kex2p activity. This activity separates from the Kex2p activity of SpaK. **Figure 5-6** shows that SpaK activity colocalizes with CPY (a soluble vacuolar marker) activity whereas tribrid protein activity does not. These results suggest that the tribrid protein is found in the Golgi and not in the vacuole. It

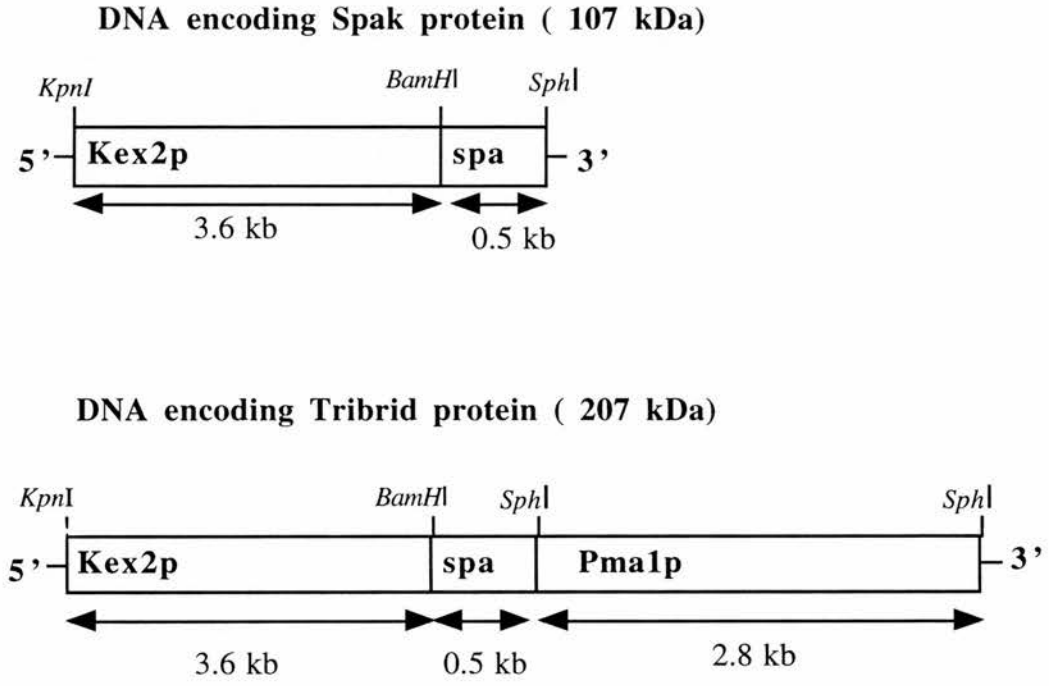


Figure 5-1 Schematic drawing of DNA encoding SpaK and the Tribrid protein.

The plasmid backbone for both constructs was YCplac22 (Gietz and Sugino, 1988). Diagnostic restriction sites and sizes are indicated. The SpaK protein is known to localize to the vacuole. Pma1p was added to produce a tribrid protein and examine the effect on the localization of SpaK.

Figure 5-2 Detection of the tribrid fusion protein.

100 ml cultures of JRY188 and of 2 pSpaKP transformants were grown to an OD_{600} of about 1. Whole cell yeast extracts were produced by glass bead lysis. After a clearing spin (500 g, 5 min) the extracts were spun at 100 000 g for 30-60 min to produce a pellet containing membranes (P100), and a supernatant containing the cytoplasm (S100). Samples of these fractions were corrected for OD_{600} and analysed on a 9% SDS-PAGE gel. 15 µg of protein was loaded per lane. After electrophoresis the gel was western blotted and a rabbit anti-bovine IgG-HRP conjugate used to detect the protein A part of the tribrid protein. The blot was developed using the ECL protocol. **Lanes 1** and **2**: JRY188(pSpaKP) P100 and S100 respectively. **Lanes 3** and **4**: P100 and S100 from a second JRY188(pSpaKP) transformant. **Lanes 5** and **6**: P100 and S100 from JRY188.

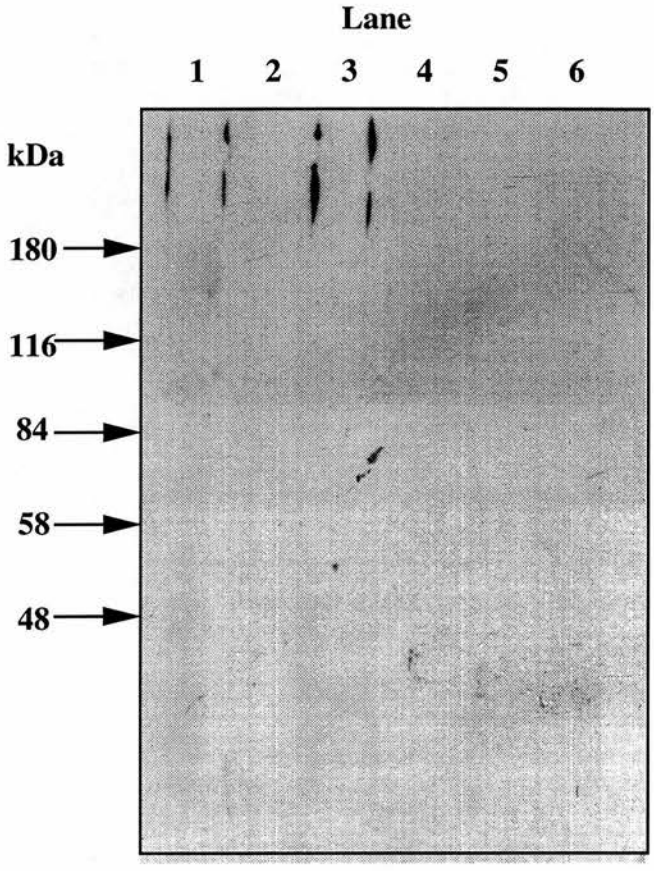


Figure 5-2: detection of the tribrid fusion protein.

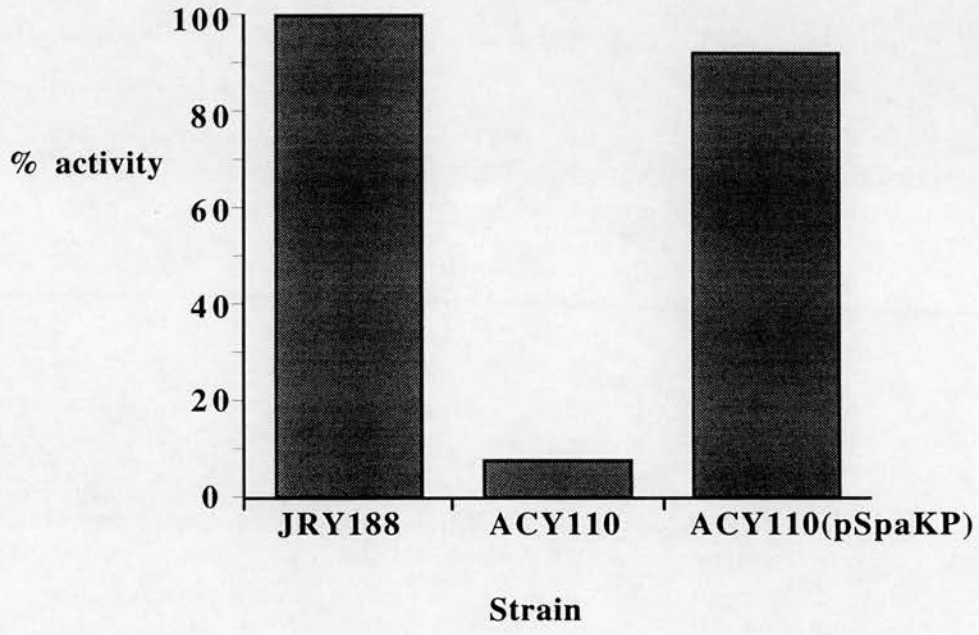


Figure 5-3 The tribrid protein has Kex2p activity. 10 ml cultures of JRY188, ACY110 (a *kex2* mutant), and ACY110(pSpaKP) were grown in minimal media to an OD_{600} of about 1. Cells were spun down for 5 min at 3000 g. Whole cell yeast extracts were produced by glass bead lysis. 50 µl samples were assayed for Kex2p activity. Activities are expressed as a % of wild type activity and have been normalized for OD_{600}.

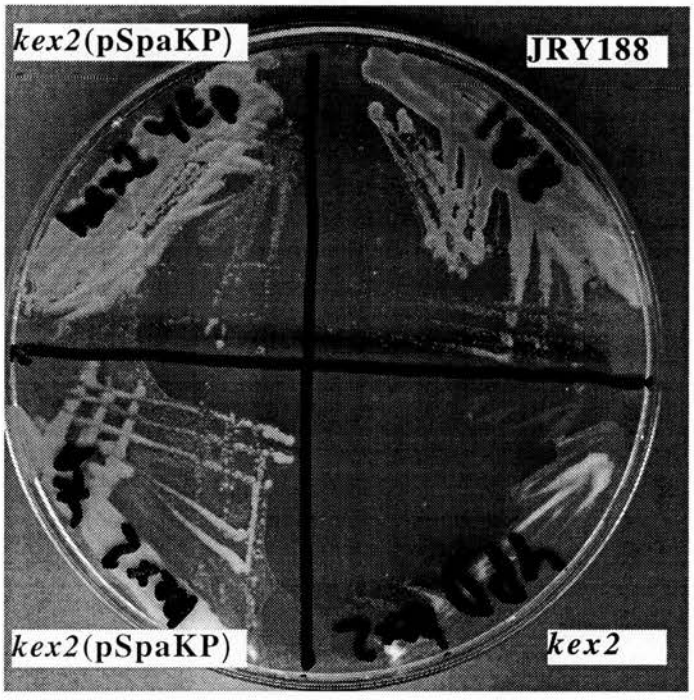


FIGURE 5-4 Expression of the tribrid protein from pSpaKP complements the cold sensitive phenotype of a *kex2* mutant. JRY188, ACY110 (a *kex2* mutant), and ACY110 transformed with pSpaKP were plated out on YPD plates and incubated in a fridge at 10°C for at least 5 days. The *kex2* mutant failed to form single colonies unless transformed with pSpaKP.

Figure 5-5 The Kex2p activity of the tribrid protein appears to have a Golgi distribution. 500 ml cultures of ACY110 (a *kex2* mutant), ACY110(pSpaK), JRY188, and ACY110(pSpaKP) were grown overnight to an OD_{600} of about 1. Cells were spun down, spheroplasts generated, and homogenised. After a clearing spin the homogenate was loaded onto a 18%-54% (w/v) sucrose gradient and spun for 2.5-h at 174 000 g. 1 ml fractions were taken and stored at -20^{o}C until required for enzyme assays. Fraction 1 is from the top of the gradient and fraction 12 from the bottom. 50 µl aliquots from each fraction were used in the Kex2p assay, units of enzyme activity are arbitary and corrected for OD_{600}.

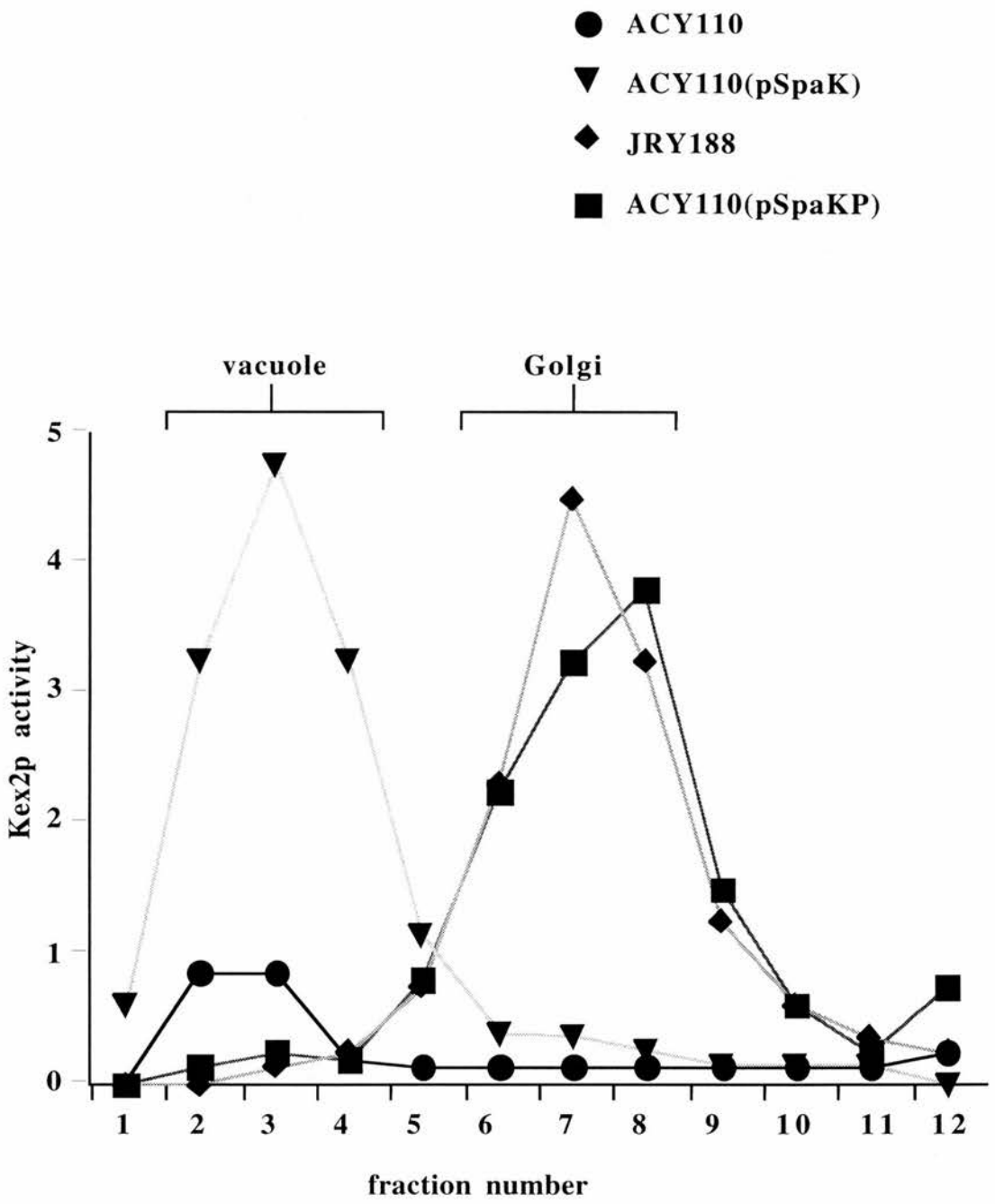


Figure 5-5 The Kex2p activity of the tribrid protein appears to have a Golgi distriution.

Figure 5-6 The Tribrid protein does not colocalise with CPY a soluble vacuolar marker. 500 ml cultures ACY110(pSpaK), and ACY110(pSpaKP) were grown overnight to an OD_{600} of about 1. Cells were spun down, spheroplasts generated, and homogenised. After a clearing spin the homogenate was loaded onto a 18%-54% (w/v) sucrose gradient and spun for 2.5-h at 174 000 g. 1 ml fractions were taken and stored at -20°C until required for enzyme assays. Fraction 1 is from the top of the gradient and fraction 12 from the bottom. 50 µl aliquots from each fraction were used in the Kex2p and CPY assays, units of enzyme activity are arbitary and corrected for OD_{600}.

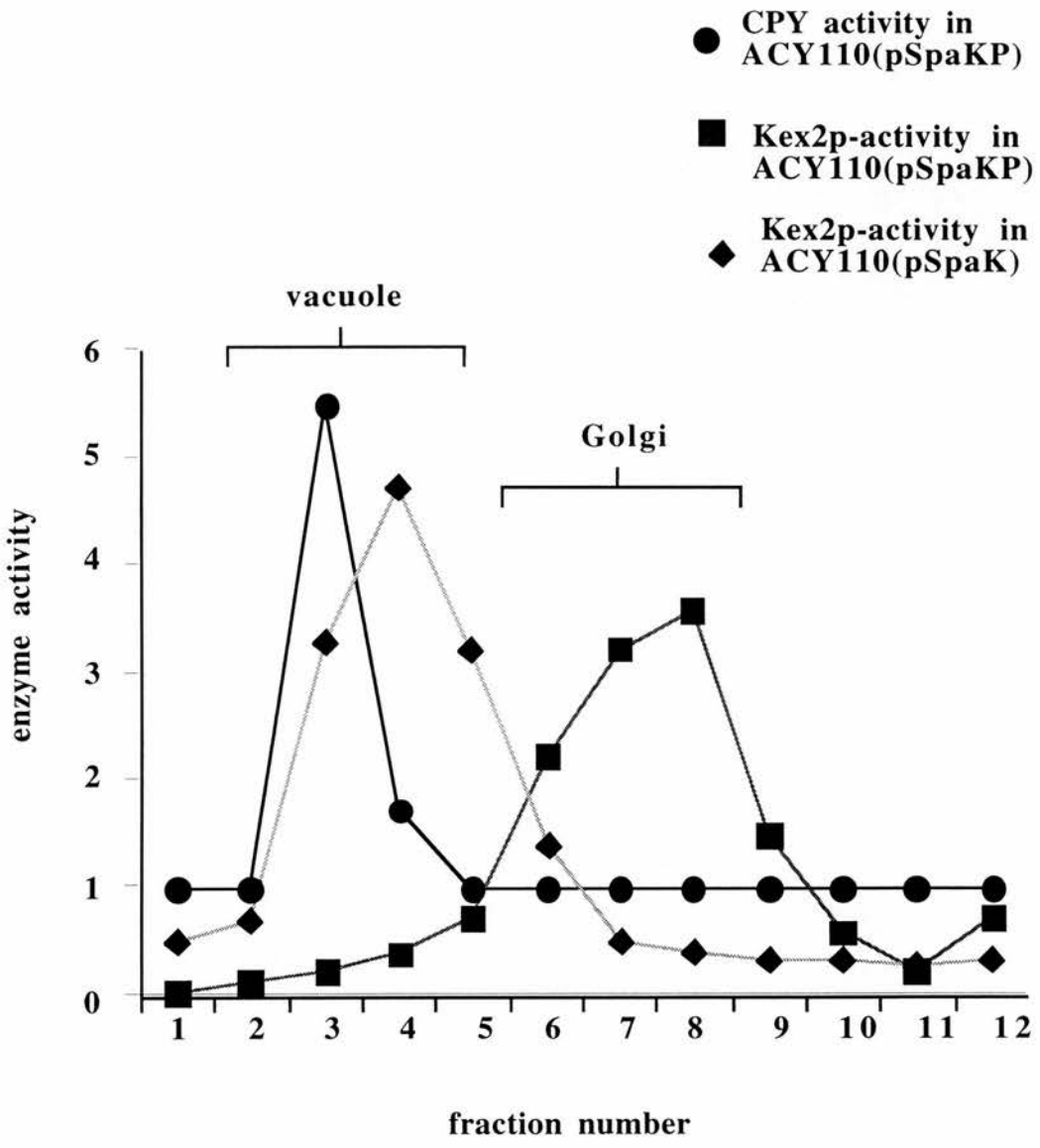


Figure 5-6: the tribrid protein does not colocalise with CPY a vacuolar marker.

would seem that addition of Pma1p sequence to SpaK has resulted in retention in the Golgi rather than transport to the vacuole. They also suggest that the addition of Pma1p has not resulted in targeting to the plasma membrane.

5-5 The tribrid protein does not colocalize with Ste2p a plasma membane protein

Ste2p is the receptor for the mating pheromone α-factor (Burkholder and Hartwell, 1985; Sprague and Thorner, 1992) and is only expressed in **a** strains. An **a** strain *kex2* mutant (ACY111) was transformed with YCpTribrid and the localization of Ste2p and the tribrid protein examined. **Figure 5-7** shows that the tribrid protein Kex2p activity does not colocalize with Ste2p although there is a slight overlap (please note that the sucrose gradients described in **figures 5-5** and **5-6** are w/v and that of **figure 5-7** w/w. This explains the difference in profiles. This does not affect the conclusions drawn in this chapter). This evidence confirms that the tribrid protein is not found in the plasma membrane and supports the proposal that the most likely location for the protein is in the Golgi.

There are many explanations why addition of Pma1p to SpaK has not directed it to the plasma membrane. One is that addition of the Pma1p sequence has restored the ability of the Golgi retention signal in the Kex2p sequence to interact with its receptor. If this retention signal was dominant over a plasma membrane signal then the tribrid protein would remain in the Golgi. The *PMA1* gene was amplified by PCR and there is a possibility that a PCR error has destroyed the putative plasma membrane targeting signal or even changed it to a Golgi retention signal. Indeed a criticism of the work presented here is that the presence of Pma1p has not been confirmed either by antibody or activity.

Figure 5-7 The tribrid protein does not colocalise with Ste2p a plasma membrane protein. A 500 ml culture of ACY111(pSpaKP) was grown in minimal selective media overnight to an OD_{600} of about 1. Cells were spun down, spheroplasts generated, and homogenised. After a clearing spin the homogenate was loaded onto an 18%-54% (w/w) sucrose gradient. After spinning for 2.5-h at 174 000 g 1 ml fractions were taken. 600 µl of each fraction was diluted 2 fold with 10 mM HEPES pH 7.5 and subjected to TCA precipitation. The rest of the fraction was stored at -20°C until required for enzyme assays. After precipitation the sample was spun for 10 min at 10 000 g and a sticky white pellet formed. Due to sucrose precipitating this pellet often proved difficult to resuspend. The pellet was resuspended in SDS-PAGE sample buffer heated for several minutes at least (up to 20 min to dissolve the sucrose) and loaded onto a 10% SDS-PAGE gel. After electrophoresis western blotting was performed and affinity purified rabbit anti-Ste2p (supplied by Dr. N. Bryant, 1/100 dilution) used to detect Ste2p. The secondary antibody was donkey-anti-rabbit IgG HRP conjugate (1/5000 dilution) ECL detection protocol was used. CPY (a vacuolar marker), and Kex2p (Golgi marker) activities were assayed in each fraction. Assays were carried out as described. 50 µl aliquots from each fraction were used in the enzyme assays. Activity units are arbitrary.

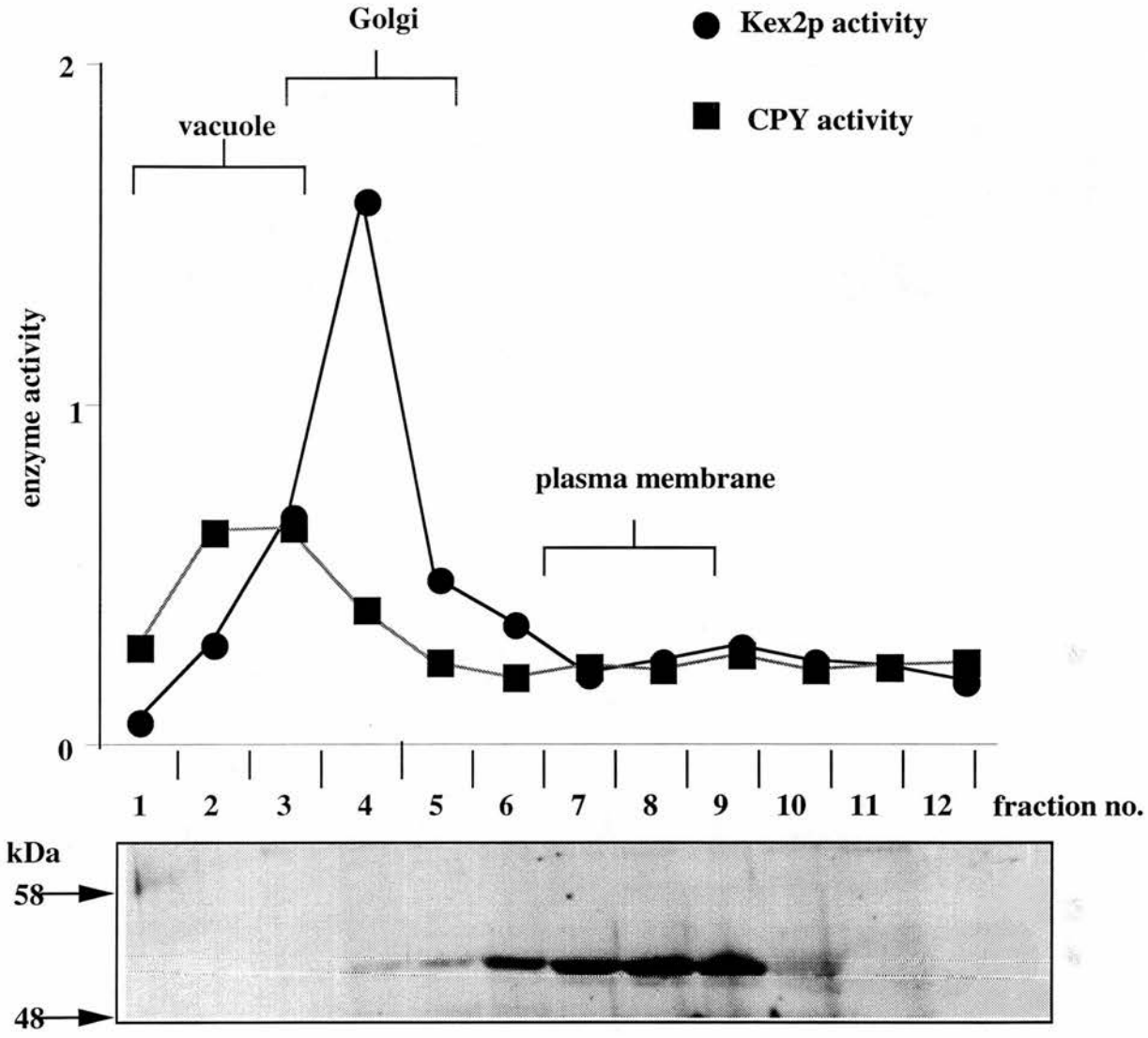


Figure 5-7: the tribrid protein does not colocalise with Ste2p, a plasma membrane protein.

An attempt to determine whether the tribrid protein had Pma1p activity was attempted. The *PMA1* gene is essential (Serrano *et al.*, 1986), however Cid *et al.*(1987) constructed a strain in which the constitutive promoter of the ATPase gene was replaced by a galactose dependent promoter. This strain is unable to grow on medium lacking galactose and develops a characteristic morphology after growth arrest (enlarged mother cell with 4 to 7 daughter cells attached). If the tribrid protein demonstrated complementing activity and allowed growth of this strain in the absence of galactose it would demonstrate that it not only had Pma1p activity but that it was localized to the plasma membrane. Ramon Serrano supplied plasmid pRS-61 (Cid *et al.*, 1987) to allow construction of a suitable strain with the Gal-controlled *PMA1* ATPase gene. However despite numerous attempts transformants could not be produced in either JRY188 or BJ5464. In light of previous results it seems unlikely that the tribrid protein would have allowed growth on glucose.

Evidence is accumulating that supports the model that transport to the vacuole is the default pathway for yeast membrane proteins, indeed the results presented in this chapter 4 support such a model. The model predicts the existence of targeting signals in plasma membrane proteins and the experiments described in this chapter were carried out to try and identify such a signal. A protein A-tagged Kex2p which mislocalized to the vacuole was fused to the Pma1p, a plasma membrane H^+ATPase. This tribrid fusion protein did not localize to the plasma membrane as hoped but instead was retained in the Golgi. Plasma membrane targeting signals have yet to be described but mutational analysis of plasma membrane proteins should reveal important sequences. One possible screen would be to make mutations in the α-factor receptor Ste2p. Mutations which prevent Ste2p targeting could be detected using a mating assay where the **a** strain expressing Ste2p is mated with an α strain. If the assay is set up so that only diploids can grow on the selective plates mating can be

easily monitored. One problem with such an approach would be to distinguish between mutations which effect targeting and those which effect receptor function. Bryant and Boyd (personal communication) have constructed a Ste2p-Pep12p fusion protein and this protein localizes to the plasma membrane. Pep12p encodes a t-SNARE and expression of the fusion protein results in CPY secretion, presumably because v-SNAREs in vacuolar-targeted vesicles can now fuse with the plasma membrane. Leucine auxotrophs which secrete CPY can grow on plates containing the peptide N-carbobenzoxy-Lphe-Lleu (CBZ-phe leu) (Stevens *et al.*, 1986) and this could form the basis of an assay for fusion protein delivery to the plasma membrane. Mutations which altered plasma membrane targeting would prevent growth on media containing CBZ-phe-leu since CPY would no longer be secreted. Selection of transformants which can grow on plates containing leucine but cannot grow on plates containing CBZ-phe-leu should select for mutations in the targeting signal.

5-6 Summary

1) Addition of the plasma membrane protein, Pma1p, to a protein A tagged-Kex2p (SpaK) prevents transport to the vacuole.

2) Subcelluar fractionation shows the tribrid protein most likely has a Golgi localisation.

3) Failure of the tribrid protein to target to the plasma membrane means this construct cannot be used to identify any potential targeting signals.

When protein A is added to the C-terminus of Kex2p the fusion protein localizes to the vacuole, supporting the vacuolar default model for membrane proteins in *S. cerevisiae*. Addition of a plasma membrane protein sequence (Pma1p) to such a vacuolar targeted protein was expected to result in transport to the plasma membrane due to targeting sequences. Instead the tribrid protein localized to the Golgi, perhaps because addition

of Pma1p sequence had restored the ability of the Kex2p retention signal to be recognized by receptors in the Golgi.

Chapter 6
Expression of a heterologous protein from *Kluyveromyces lactis*

6-1 Introduction

S. cerevisiae has been extensively used as a host for the production of heterologous proteins in both industrial and pure research situations (reviewed in Romanos *et al.*, 1992; Fleer, 1992; Buckholz and Gleeson, 1991) and from an industrial point of view *S. cerevisiae* has many advantages. *S. cerevisiae* has been used in the brewing and food industries for hundreds of years and has acquired GRAS status (Generally Regarded As Safe) and if heterologous proteins are to be used in medical situations it is important they are produced in a safe way. Being a unicellular microorganism which is very easy to grow in large cultures, production of commercially viable amounts of protein possible, another advantage of *S. cerevisiae*.

S. cerevisiae is a eukaryotic organism and has a cellular organisation similar to animal cells allowing heterologous proteins to be targeted into a secretory pathway. Secretion of a protein through a multi-compartment secretory pathway is often essential for proper post-translational modifications to occur. This is of great importance when a protein as authentic as possible is required. Secretion also brings the advantage of easier product purification since yeasts secrete very little protein into their growth media. *S. cerevisiae* has however been found to have several problems as a host for protein expression. A major problem has been poor yield, even when a strong promoter is used, and the reduction in plasmid stability when proteins are stressful to the yeast. The ability to genetically manipulate *S. cerevisiae* easily has been exploited to overcome these problems. One approach is to include an autoselection system in the plasmid carrying the heterologous gene. For example the *SRB1* gene is required for osmotic shock stability in *S. cerevisiae* (Stateva *et al.*, 1991). *srb1* mutants transformed with a plasmid carrying a wild type *SRB1* gene grow normally in non-stabilized media but lyse if the plasmid is lost. By including an *SRB1* gene on an expression plasmid and by growing the cells in appropriate media, an autoselection

mechanism for the plasmid results (Rech *et al.*, 1992).

Another approach to improving yields is to screen for 'super-secreting' strains. Smith *et al.*(1985) employed a mutagenesis approach coupled with a rapid plate assay which detected secreted prochymosin to isolate super-secreting mutants. They found that in normal cells less than 1% of the bovine prochymosin was secreted but that in certain *ssc* mutants secretion was greatly improved. The *ssc1* strain was able to secrete 5-50 fold higher levels of several heterologous proteins. The *SSC1* gene has since been identified, renamed *PMR1*, and is thought to encode a Golgi Ca^{2+}ATPase (Rudolph *et al.*, 1989; Antebi and Fink, 1992).

Post-translational glycosylation in yeast differs from that in animal cells and this can cause problems with protein authenticity. In animal cells mannose residues are removed and additional complex sugars added in the Golgi complex. In contrast, *S. cerevisiae* lacks Golgi mannosidases and instead mannose residues are added (Romanos *et al.*, 1992). In this way hyperglycosylation occurs and can effect immuogenicity and enzyme activity of heterologous proteins. Again this problem has been addressed by identification of mutants such as those which are defective in mannan biosynthesis (*mnn*) (Kukuruzinska *et al.*, 1987).

More recently the availability of transformation technologies in non-*Saccharomyces* yeasts has allowed expression of heterologous proteins in several other species to be investigated. In nearly all cases tested the yeasts *Pichia pastoris*, *Hansenula polymorpha*, *Yarrowia lipolytica*, and *Kluyveromyces lactis* secrete heterologous proteins at considerably higher levels that *S. cerevisiae* (Buckholz and Gleeson, 1991). For example *K. lactis'* great promise as a host for protein production was demonstrated when 95% of bovine prochymosin was secreted compared with 1% in

S. cerevisiae (van den Berg *et al.*, 1990). Why *K. lactis* has this increased capacity in its secretory pathway is not clear but prochymosin from *K.lactis* is now in commercial production. In this system the bovine prochymosin gene was integrated into the genome because of worries about plasmid stability. Fleer *et al.* (1991a) have since demonstrated that plasmid derivatives of pKD1 (Bianchi *et al.*, 1987; Chen *et al.*, 1989), a circular plasmid isolated from *Kluyveromyces drosophilarum*, are extremely stable in *K. lactis*. They showed that under non-inducing conditions, a pKD1-based plasmid carrying a recombinant human serum albumin gene was present in 90% of cells after 100 generations in non-selective media. A pKD1 based plasmid has also been used to express human interleukin-1β in *K. lactis* and a 80-100 fold increase in levels compared with *S. cerevisiae* were reported (Fleer *et al.*, 1991b). pKD1 based plasmids are not only stable but increase the gene dosage which is often important for high levels of expression. Another way to stably increase gene dosage is to use a high-copy number intergration system. Bergkamp *et al.* (1991) have developed a vector system which allows high-copy number integration into the ribosomal DNA of *K.lactis*. Using this system the gene for a plant α-galactosidase was expressed and the obtained high levels of protein secretion. The strains they produced contained various numbers of integrated plasmid, 15 copies being the maximum and producing highest levels. On the basis of these reports of successful protein secretion from *K. lactis* a pKD1-based plasmid was constructed.

This chapter outlines the construction of an episomal expression vector for use in *K. lactis*. The vector was constructed to allow a comparison of secretion of proteins from *K. lactis* compared with *S. cerevisiae*. The test protein for this study was human elafin, an elastase inhibitor. The strategy for construction was to create a YIplac211 (Geitz and Suigino, 1988) derivative carrying a promoter-signal-terminator cassette into which the elafin gene was cloned. The *LAC4* promoter was chosen because it is a

strong inducible promoter. In the presence of lactose or galactose the levels of intracellular β-galactosidase increase 20 to70 fold (Das *et al.*, 1985; Leonardo *et al.*, 1987). The use of this promoter should allow high levels of expression of heterologous proteins and for these levels to be controlled by the presence of lactose or galactose in the growth medium. This is of great use if the protein being expressed is deleterious to yeast growth. The signal sequence used to direct proteins into the secretory pathway was from the *ORF2* gene of *K. lactis*. This encodes the α subunit of the *K. lactis* toxin (Stark and Boyd, 1986; Stark *et al.*, 1990) and is known to direct efficient secretion of heterologous proteins from *K. lactis* (Fleer *et al.*, 1991a). The terminator from the *ADH1* gene of *S. cerevisiae* was used to promote efficient termination of transcription. Finally the pKD1 sequence was introduced at a vector unique restriction site. pKD1 allows the plasmid to replicate autonomously and appears to have a functional organisation analogous to that of the 2μ DNA of *S. cerevisiae* (Bianchi *et al.*, 1987; Chen *et al.*, 1989).

6-2 Cloning a *LAC4* promoter fragment

A functional promoter fragment was isolated from plasmid pKR1B*LAC4*del*Xma*I as follows. pKR1B*LAC4*del*Xma*I was digested with *Eag*I and this site was filled in. The plasmid was then digested with *Bcl*I and the *Eag*I/*Bcl*I fragment gel purified and cloned into the *Sma*I and *Bam*HI sites of M13mp18. No suitable restriction sites were available at the 3' end of this fragment into which the *ORF2* signal sequence could be cloned. A *Cla*I site was introduced at position -17 of the *LAC4* promoter to accept the *ORF2* DNA. This was done by site-directed mutagenesis using the Klunkel method (Klunkel *et al.*, 1991). Oligonucleotide 790K was used to introduce this site. The sequence of 790K and the *LAC4* DNA to which it anneals is shown below. The *Cla*I site is underlined.

790K 5' CTT TCA GTT ATC GAT GAG T 3'

LAC4 3' GAA AGT CAA GAG CTA CTC A 5'

This mutation was confirmed by DNA sequencing. The [*Eag*I]/*Bcl*I fragment contains an *Eco*RI site which was eliminated to allow pKD1 to be introduced at a unique *Eco*RI site later in the vector construction. An *Sst*I/*Hin*dIII fragment containing the *LAC4* promoter was cloned into a similarly digested derivative of pK18 called pK18[EcoRI]. The *Eco*RI site in this pK18 derivative had previously been destroyed by filling in. This meant the only remaining *Eco*RI site was in the *LAC4* promoter and this was destroyed by digestion followed by filling in. The *Sst*I/*Hin*dIII fragment was then sub-cloned from pK18[EcoRI] into a YIplac211 derivative which has had the *Stu*I site in the *URA3* gene destroyed (see below). In summary, a fragment of the *LAC4* promoter was cloned and a *Cla*I site introduced to accept the *ORF2* signal sequence. An *Eco*RI site was also been destroyed.

6-3 Cloning the *ORF2* signal sequence

The *ORF2* signal sequence was amplified by PCR using oligonucleotides 1300/92 and 1301/92 (see **table 6-1**). Template DNA was pKla102() and Taq polymerase was used. Amplification was carried out for 20 cycles of 1 min 95°C, 1 min 55°C, 1 min 72°C. The PCR product was cloned as a *Cla*I/*Sal*I fragment into the YIplac211 derivative. DNA sequencing of both strands revealed two errors in the PCR product. One error was within the primer 1300/92 and upon checking the sequence of the primer a mistake was found. A new 3' primer was synthesized, oligonucleotide 1690/92. The *ORF2* signal was reamplified using primers 1300/92 and 1690/92. DNA sequencing again showed that the same PCR error had occured within the *ORF2* sequence. This error was deletion of a T base. PCR was repeated using various reaction conditions but this error was always present. However when $Vent_r$

Table 6-1. Details of primers used for PCR of *ORF2* DNA

Shown above the table is the nucleotide sequence of the ORF2 target DNA used in the PCR reactions described.

In cloning of the *LAC4* promoter the sequence between the ATG codon and the introduced *Cla*I site was deleted. When the *ORF2* DNA was amplified the 5' primer, 1300/92 was designed to replace this lost sequence. The ATG codon is shown outlined and the *Cla*I site is underlined.

The 3' primer for the *ORF2* DNA, 1301/92 has *Sal*I (GTCGAC) and *Stu*I (AGGCCT) sites (underlined). The *Sal*I site allows cloning of the PCR product. Genes to be expressed are cloned blunt ended into the *Stu*I site. The *KEX1* gene product cleaves proteins after KR (Wesolowski-Louvel *et al.*, 1988). The *Stu*I site AGG sequence encodes R, therefore a proper processing site is produced. This primer has a nucleotide missing and primer 1690/92 had to be synthesized.

Primer 1690/92 has a *Stu*I site to allow cloning of the 1300/92, 1690/92 PCR product as a *Cla*I/*Stu*I fragment. It corrects the mistake in primer 1302/92.

Table 6-1. Details of primers used for PCR of *ORF2* DNA.

Sequence of DNA encoding the *ORF2* signal

5'AAAATGAATATATTTTACATATTTTTGTTTTTGCTGTCATTCGTTC

AAGGTTTGGAGCATACTCATCGAAGAGGCTCCTTAGTCAAAAGA 3'

Primer name	Primer sequence
5' Primer 1300/92	5'CCATCGATAACTGAAAGATATGAATATATTTTA CATATTTTTGTTTTTGC 3'
3' Primer 1301/92	5'GCGGTCGACAGGCCTTTTGACTAAGAAGCCTC TTCG 3'
3' Primer 1690/92	5'AAAAGGCCTTTTGACTAAGGAGCC 3'

Polymerase (New England Biolabs) was used instead of Taq the error was not produced. The PCR product was cut with *Cla*I and *Stu*I and cloned into the vector containing the incorrect *ORF2* sequence which was removed by digestion with *Cla*I and *Stu*I.

6-4 Cloning an *ADH1* terminator into the vector

An *ADH1* terminator from pDP316 (provided by Dr. D. Pioli) was cloned as a *Xho*I/*Sal*I fragment into the *Sal*I site of the YIplac211 derivative. Orientation of this fragment was confirmed by DNA sequencing using oligonucleotide 1858/92 as a primer;

1858/92 5' CCTCTGGCGAAGAAGAAGTCC 3'

6-5 PCR mutagenesis to destroy the *Stu*I site in the *URA3* gene of YIplac211

Genes to be expressed in pAC1 must be cloned into the *Stu*I site at the 3' end of the *ORF2* DNA sequence. This maintains a Kex1p processing site in the translated protein which facilitates the removal of the *ORF2* signal sequence (Wesolowski-Lovel *et al.*, 1988). YIplac211 contains a *Stu*I site and this had to be destroyed. This was done by PCR mutagenesis as follows. Two PCR reactions were performed, the first amplified *URA3* DNA between the *Nsi*I site and the *Stu*I site using primers 1814/92 and 1812/92. The second reaction amplified the DNA between the *Stu*I site and the *Nco*I site using primers 1811/92 and1813/92. Both reactions were carried out for 10 cycles only using $Vent_r$ Polymerase. This was to minimize the chance of PCR errors.

1811/92 5' ACATCAAAAGGGCTCTAGGTTCCT 3'

1812/92 5' AGGAACCTAGAGCCCTTTTGATGT 3'

1813/92 5' GATTTTTCCATGGAGGGCACAGTTAAGCC 3'

1814/92 5' TAGTATACATGCATTTACTTATAATAC 3'

Primers 1812/92 and 1813/92 are complementary and alter the *Stu*I site sequence from AGGCCT to AGCCCT. This maintains the correct amino acid sequence upon translation. After these two PCR reactions had been performed the products were gel purified. These two fragments encompassing the mutation were annealed with each other and extended by mutually primed extension using the flanking primers 1813/92 and 1814/92 (20 cycle of 95°C 1 min, 60°C 1 min, 72°C 1 min). The result was a full length *Nsi*I/*Nco*I fragment of the *URA3* gene with the *Stu*I site destroyed. This fragment was cloned after digestion with *Nsi*I and *Nco*I into YIplac211. DNA sequencing revealed that the *Stu*I site had been destroyed and that a PCR error had resulted in deletion of a T base. Mutagenesis could have been repeated but time considerations resulted in a strategy whereby genes were cloned into the *Stu*I site in the expression cassette and this cassette was then cloned into a YIplac211 plasmid with a fuctional *URA3* gene.

6-6 Introduction of the elafin gene and pKD1 sequence

The elafin coding sequence was amplified using primers 1562/92 and 1563/92:

1562/92 5' GCTCAAGAACCAGTTAAAGGT 3'

1563/92 5' CGGGATCCTATTATTGTGGAA 3'

Template DNA was pDP290(provided by Dr. D. Pioli). 20 cycles of 95°C 1 min, 55°C 1 min, 72°C 1 min using $Vent_r$ Polymerase were carried out. DNA sequencing of both strands revealed no PCR errors. The expresssion cassette was cloned as an *Sst*I/*Hin*dIII fragment into a YIplac211 vector with a functional *URA3* gene. pKD1

was cloned from pCXJ-Kan1(Chen *et al.*, 1989) as a 4.7kbp *Eco*RI fragment and ligated into the *Eco*RI site of YIplac211. This was the final step in the construction of the plasmid. The completed plasmid containing the elafin gene was named pAC2 and the plasmid containing no gene pAC1. **Figure 6-1** shows a schematic of the finished plasmid.

6-7 Stability of pAC1 and pAC2

To check if instability of pAC1 and pAC2 would be a problem, cultures of the *K. lactis* strain MW98-9C (Fleer *et al.*, 1991) transformed with pAC1, pAC2, and pCXJ-Kan1 were grown to an OD_{600} of about 2 in non-selective medium (YPD). **Table 6-2** shows that pAC1 is as stable as pCXJ-Kan1 and that cloning in the elafin gene does not affect stability. Futhermore no dramatic loss of plasmid or growth problem was observed when elafin expression was induced by addition of lactose to the growth medium. These qualitative experiments suggest that the pKD1-based plasmids pAC1 and pAC2 are stable in *K. lactis*. This agrees with previous observations (Bianchi *et al.*, 1987; Chen *et al.*, 1989; Fleer *et al.*, 1991).

6-8 Elafin assay calibration curve

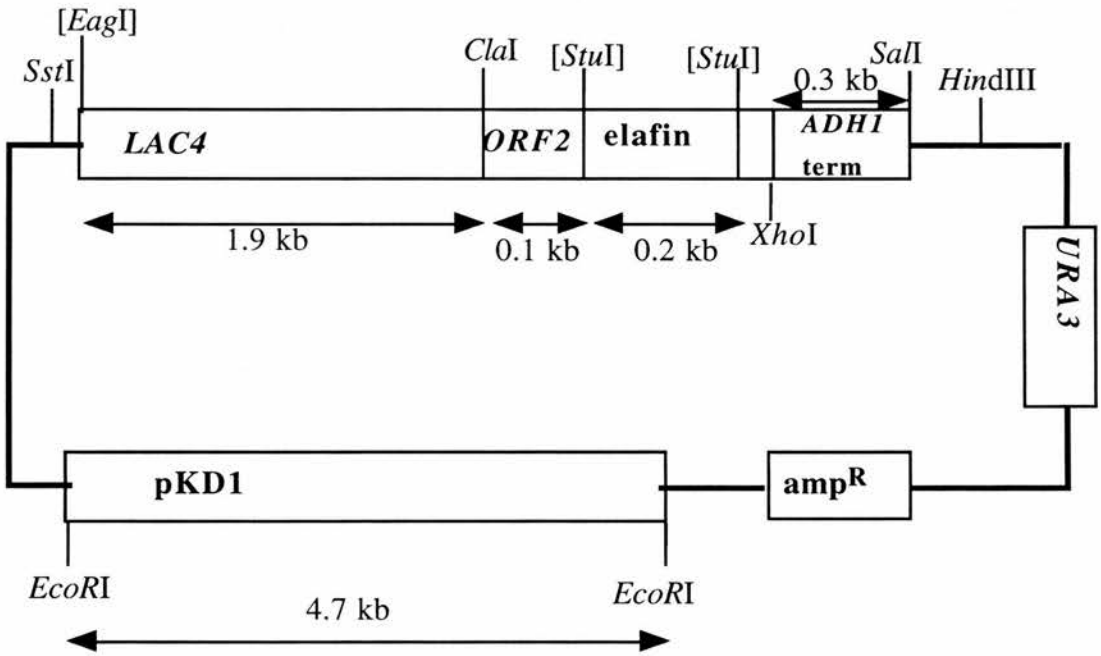
In order to estimate the levels of secreted elafin from *K. lactis* and *S. cerivisiae* a calibration curve was generated for an elastase inhibition assay. The lower the activity of elastase in this assay the more elafin present in the sample. Purified human elafin (supplied by Dr. D. Pioli) was used in the elastase inhibition assay.
Figure 6-2 shows the calibration curve generated and this curve is in agreement with curves generated by Zeneca Pharmaceuticals (data not shown).

Figure 6-1. Schematic of plasmid pAC2

The figure shows the major components of the plasmid pAC2 and a brief summary of construction is outlined below.

1) The *LAC4* promoter was isolated and modified.
2) *ORF2* signal sequence DNA was amplified by PCR.
3) A *LAC4-ORF2* cassette was generated in YIplac211 which carries a *URA3* selectable marker. The *Stu*I site in this marker has been destroyed.
4) PCR errors in the *ORF2* and *URA3* gene are found.
5) A new *ORF2* PCR product is exchanged for the old one.
6) An *ADH1* terminator is cloned into the *LAC4-ORF2* cassette.
7) The elafin gene is amplified by PCR and cloned into a unique *Stu*I site at the 3' end of the *ORF2* signal sequence.
8) The whole cassette containing the elafin gene is cloned into a new YIplac211 vector. This is because PCR mutagenesis of the *Stu*I site in the *URA3* gene made it non-functional.
9) The cassette is now in a plasmid with a functional selectable marker and the final step is the introduction of pKD1. pKD1 is introduced at a unique *Eco*RI site and allows the plasmid to replicate in *K. lactis*.

Figure 6-1. Schematic of plasmid pAC2



plasmid	pAC1	pAC2	pCXJ	none	pAC1	pAC2	pCXJ	none
carbon source	glu	glu	glu	glu	lac	lac	lac	lac
no. colonies	364	352	402	0	404	328	362	0

Table 6-2 Stability of pAC1 and pAC2 in the *K. lactis* strain MW98-9C.

10 ml cultures of MW98-9C transformed with the plasmids indicated above were grown overnight in selective media. These precultures were used to innoculate duplicate 100ml flasks of YPD for each culture. Culture were grown to an OD_{600} of about 0.7. One of each duplicate culture was harvested (3000 g, 5 min) washed in sterile water and added to 100 ml of YPL (containing lactose). All culture were left to grow to an OD_{600} of between 10 and 15. Dilutions from each culture were made and plated out on selective media lacking uracil. Only cells harbouring a plasmid will be able to grow. Plates were left to grow for 4 days and then the number of colonies per plate estimated by dividing the plate into 4 sectors and counting the number of colonies in 1 sector. This number was then multiplied by 4. The figures shown above are normalized for OD_{600}.

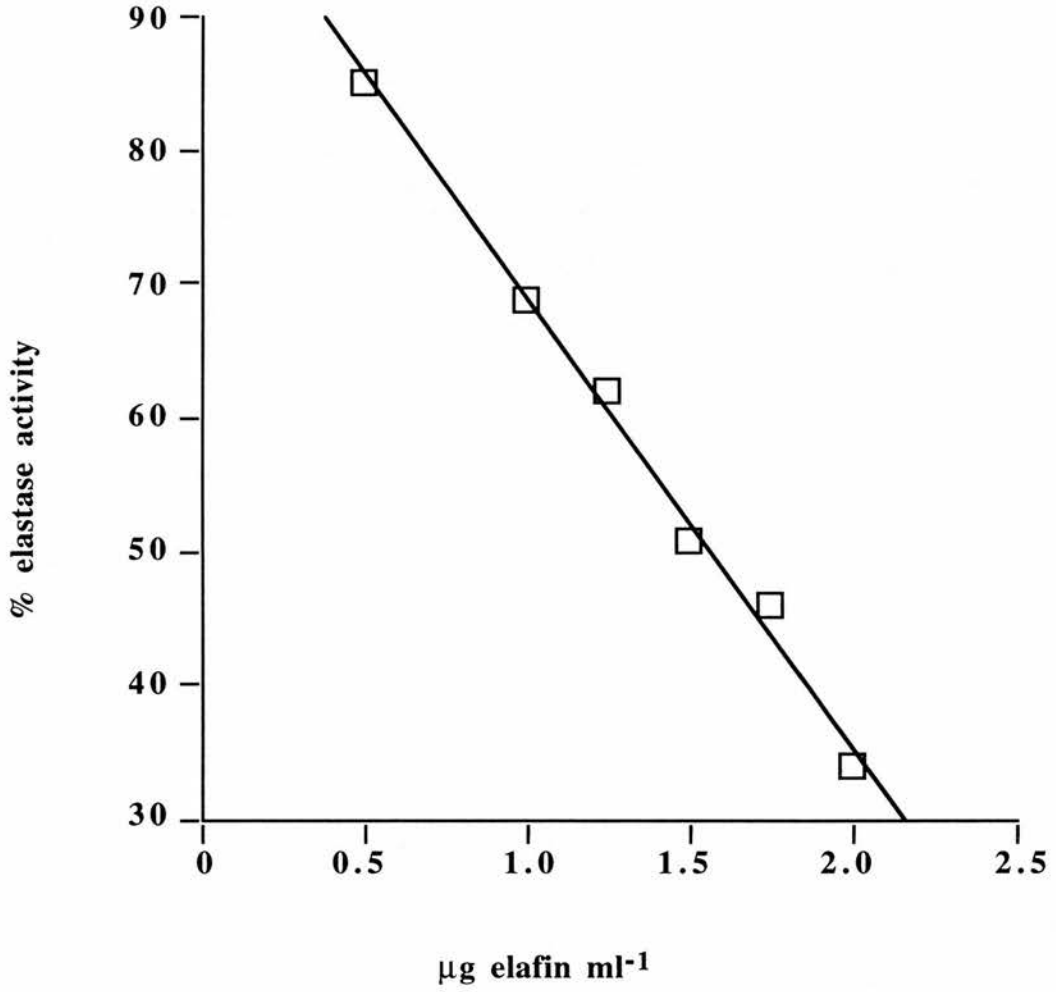


Figure 6-2 Elafin assay calibration curve.

A calibration curve was produced by assaying the % elastase activity in the presence of various amounts of purified human elafin. 100 µl samples of the various elafin dilutions were used in the assay and the graph above converts the % elastase activity into µg elafin ml^{-1} rather than µg 100 μl^{-1}. Details of this assay are described in Materials and Methods.

6-9 Comparison of elafin secretion from the *K. lactis* strain MW98-9C and the *S. cerevisiae* strain PMY1-60

MW98-9C(pAC2) and PMY1-60 were grown in non-selective media since pAC2 is stable and PMY1-60 carries an elafin expression cassette stably integrated into it's genome. MW98-9C secretes up to seven times as much elafin compared to PMY1-60 (**Table 6-3**). Maximal levels of elafin in the media are reached sometime between 18 and 24 hours growth for both strains. This is some 12 to 18 hours after MW98-C(pAC2) was induced by transfer from YPD to YPL. Levels of secreted elafin were not increased from PMY1-60 grown in YPL (data not shown).

6-10 Summary

1) A stable episomal plasmid was constructed for expression and secretion of heterologous proteins from *K. lactis*.

2) Biologically active human elafin (an elastase inhibitor) was successfully secreted from *K.lactis* strain MW98-9C.

3) The level of secreted elafin was upto seven-fold higher from *K. lactis* than from a *S. cerevisiae* strain in shake flask cultures.

In conclusion the data shown in this chapter demonstrates the potential usefulness of *K. lactis* as an alternative host to *S. cerevisiae* for the expression of heterologous proteins. For the one test protein example used, human elafin, the amount of secreted protein was significantly higher.

strain	MW98-9C (pAC2)	MW98-9C (pAC2)	PMY1-60	PMY1-60
time				
6 h	0.5	0.4	0.1	0.4
18 h	6.1	6.5	0.8	1.2
24 h	6.4	7.6	1.3	1.1
48 h	5.3	6.2	0.9	0.6

Table 6-3 Elafin secretion from MW98-9C (*K. lactis*) and PMY1-60 (*S. cerevisiae*). 100 ml cultures of MW98-9C was grown to an OD_{600} of about 0.6 in YPD before the cells were harvested (3 000 g, 5 min) washed once in sterile water and resuspended in YPL. The lactose in YPL induces the *LAC4* promoter and elafin expression. PMY1-60 expresses elafin constituitvly from *MFα1* promoter and was grown in YPD for the whole experiment. Samples of culture supernatent were taken at the times indicated and assayed for elafin activity as described in the material and methods section. Dilutions were necessary for supernatent from the MW98-9C strain and the amount of elafin estimated using the standard curve shown in **figure 6-2.** Units are µg elafin ml^{-1} culture supernatent.

Chapter 7
Discussion

Discussion

Mutations in the *PMR1* gene of *S. cerevisiae* produce a complex Ca^{2+}-sensitive phenotype affecting the secretory pathway and in particular Golgi function. Experiments were carried out to try and determine the cause of these defects. The results presented in chapter 3 of this thesis suggest that the Ca^{2+}ATPase Pmr1p is required for normal Golgi organization in *S. cerevisiae. pmr1* mutants appear to mislocalize Golgi enzymes and this can explain most of the *pmr1* phenotype. The approach used to study Golgi protein localization was to isolate the Kex2p-containing organelles from the homogenates of *PMR1* and *pmr1* strains. This was done using affinity-purified anti-Kex2p antibodies attached to a matrix which was incubated with yeast homogenates. When Kex2p-containing vesicles were isolated from a *PMR1* strain the fraction contained 25% DPAP A activity and no GDPase activity. This result agrees with that of Bryant and Boyd (1993). However when Kex2p-containing vesicles were isolated from a *pmr1* strain DPAP A no longer colocalized with Kex2p and that 50% of GDPase activity was now recovered. What this approach fails to distinguish is whether Kex2p is mislocalized or whether DPAP A and GDPase have mislocalized. Loss of integrity of Golgi subcompartments could also explain the altered enzyme localization if for example Golgi subcompartments had fused together, as is thought to happen in *sec7* mutants (Novick *et al.*, 1980; Esmon *et al.*, 1981; Segev *et al.*, 1988). It may be a combination of both these effects is responsible for the observed results and the *pmr1* phenotype. It is not obvious how these two models could be distinguished since a compartment is defined by the proteins it contains.

Another possibility is that Golgi subcompartments, usually distinct, become physically associated in *pmr1* mutants. Higher eukaryotes and the yeast *S. pombe* have their Golgi apparatus arranged in a stack of cisternae, but this is rarely seen in *S. cerevisiae* (see section **3-1**). Rothman and Warren (1994) have suggested that Golgi stacking

may be mediated by interactions between v-SNAREs and t-SNAREs in the Golgi membranes. The synaptic vesicle calcium-binding protein, synaptotagmin may be responsible for preventing premature release of neurotransmitters by clamping the SNARE complex in a fusion resistant conformation (see section **7-5**) and similar proteins may be responsible for clamping SNAREs in Golgi membranes together, the result being stacking. SNAREs have been identified in *S. cerevisiae* and, although Golgi stacking is not normally seen, it could be that abnormal Ca^{2+} concentrations in *pmr1* mutants allow these SNAREs to bind and this might allow the subcompartments containing GDPase to bind that containing Kex2p for example. One way to distinguish between a physical association model and a colocalization model would be to use reporter proteins similar to those described by Chapman and Munro (1994). They fused invertase to various mannosyltransferases which are membrane proteins involved in processing of carbohydrate side chains, and are found in the same early Golgi compartment as GDPase. Between the invertase and the mannoslytransferase the engineered a Kex2p processing site. These fusion proteins were localized normally but in certain mutants Golgi retention was disrupted and this resulted in the fusion protein being transported along the secretory pathway and being exposed to Kex2p activity in the TGN. Here invertase was released from the mannosyltransferase and the secreted invertase can be detected using a colorimetric plate assay. This assay allowed selection of Golgi retention mutants. If such a construct were introduced into a *pmr1* mutant and the Golgi compartments containing the mannosyltransferase had fused with the TGN then Kex2p would cleave the fusion protein releasing invertase. If however the compartments were only physically attached then Kex2p would have no access to the fusion protein and no invertase would be secreted.

By immunoisolating the Pmr1p-containing compartment the localization of the protein could be more exactly defined and perhaps shed some light on its fuction. In the absence of affinity-purified antibodies against Pmr1p, a protein A-tagged Pmr1p was expressed and IgG-Sepharose used to isolate membranes containing this protein. This fusion protein was mislocalized to the vacuole (chapter 4). Clearly further light could be shed on Pmr1p's function if its compartment was isolated. Pmr1p contains the same FXFXD motif in it's cytoplasmic domain as DPAP A (see **3-3 introduction**) and perhaps this indicates that Pmr1p is retained in the same compartment as DPAP A. It would be interesting to see whether overexpression of DPAP A results in transport of Pmr1p to the vacuole as a result of a competition for receptors. In class E *vps* mutants a prevacuolar compartment accumulates and DPAP A has been detected there (Raymond *et al.*, 1992; Roberts *et al.*, 1989; Roberts *et al.*, 1992) and Pmr1p might also be found in this compartment if it cycles between the TGN and prevacuolar compartment as suggested for DPAP A. Pmr1p colocalizes with Kex2p 26% of the time as judged by indirect immunofluoresence (Antebi and Fink, 1992), a number that is remarkably similar to the percentage of DPAP A activity colocalizing with Kex2p activity (Bryant and Boyd, 1993; this work). Is this indicative of DPAP A and Pmr1p being colocalized?

Further evidence for Pmr1p's localization to the TGN and for cycling between the TGN and prevacuolar compartment comes from recent work by Rudolph *et al.* (personal communication). In a screen for high copy number suppressors of *pmr1* they identified *PMC1* and *VPS10* as suppressors. *PMC1* encodes a vacuolar Ca^{2+}ATPase and it is easy to imagine how it could suppress *pmr1* mutations. It would be interesting to see if overexpression of Pmc1p results in some Golgi localization of the protein which could explain this suppression. *VPS10* encodes the receptor for p2CPY in the TGN and is thought to cycle between the TGN and prevacuolar

compartment. How might a receptor suppress a defect in a Ca^{2+}ATPase? Rudolph *et al.* (personal communication) suggest that overexpression of Vps10p increases the rate of vesicular transport between the prevacuolar compartment and the TGN. They propose that the prevacuolar compartment is rich in Ca^{2+} and that increased delivery of luminal contents from here to the TGN could suppress the *pmr1* mutation. This model is based on several experimental observations made in Rudolph's laboratory. When the large luminal domain of Vps10p was replaced with invertase it was still able to suppress *pmr1*, however the cytoplasmic tail was essential for suppression, suggesting that cycling between the prevacuolar compartment and the TGN is essential. The delivery of Pmc1p to the TGN by this route was ruled out since suppression of *pmr1* occurs in *pmr1 pmc1* double mutants. *pmr1 pmc1* double mutants are non-viable in all media (Cunningham and Fink, 1994) so it is somewhat surprising that overexpression of a receptor for p2CPY can suppress this. The involvement of the vacuolar Ca^{2+}/H^{+} antiport has yet to be investigated.

Rudolph *et al.* (personal communication) have also looked at *pmr1 end3* and *pmr1 end4* double mutants. *END3* and *END4* genes encode proteins required for internalization of α-factor and the accumualtion of lucifer yellow-CH (Raths *et al.*, 1990; Raths *et al.*, 1993) and *pmr1* mutants with these *end* mutations cannot grow properly even when Ca^{2+} is added to the growth media. Rudolph suggests that this is because the endocytic pathway is normally responsible for delivery of extracellular Ca^{2+} to the prevacuolar compartment and from here Ca^{2+} can be deliverd to the Golgi to suppress the *pmr1* phenotype.

Interest in the *pmr1* mutants was originally because of its supersecreting phenotype. However, another approach was taken to try and improve secretion of a heterologous protein, namely secretion from a non-*Saccharomyces* yeast. The final chapter in this

thesis describes the use of yeast *K. lactis* to secrete human elafin. Elafin was secreted at levels upto seven-fold higher than from a *S. cerevisiae* strain and this confirms previous reports that *K. lactis* has a high secretory capacity. This work offers no insight into why *K. lactis* should secrete elafin at higher levels. Higher levels of protein might be produced in *K. lactis* due to better plasmid stability or higher levels of transcription and translation for example. Another possibility is that the protein folds better and is more stable in the *K. lactis* environment. It could also be that the secretory pathway, or certain steps in it, is more efficient in *K. lactis* compared with *S. cereivisiae.* The work described was an initial pilot study and suggests that researchers should not only concentrate on selection of oversecreting mutants, but also test whether better results are obtainable in non-*S. cerevisiae* strains. A PCR product corresponding to the *PMR1* gene from *K.lactis* was cloned (data not shown) during the course of this work. It would be interesting to disrupt this gene and see what, if any, effect this has on secretion from *K. lactis.*

Appendix

Table A-1 ***Escherichia coli*** **strains used in this study**

Strain	Genotype	Source
NM522	*supE thi Δ(lac-proAB) hsd5 F'[proAB$^+$lacI^{q}lacZΔM15]*	Gough and Murray (1983)
DH5α	*supE44 ΔλacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco BRL
BW313	*dut$^-$ ung$^-$ F'*	M. White

Table A-2 Yeast strains used in this study

Strain	Genotype	Source
S. cerevisiae		
JRY188	*MATα leu2-3,112 ura3-52 trp1 his4 sir3 rme*	Brake *et al.*, (1984)
BJ5464	*MATα ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prbΔ1.6R can1 GAL*	Yeast Genetic Stock Centre (YGSC) Berkeley California
BJ5465	*MAT**a** ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prbΔ1.6R can1 GAL*	YGSC Berkeley California
ACY100	JRY188 *pmr1-2::URA3*	This study
ACY110	JRY188 *kex2::LEU2*	This study
ACY111	BJ5465 *kex2::LEU2*	This study
K. lactis		
MW98-9C	*α uraA argA lysA* K^+ pKD1^o	M. Stark University Dundee

A-3 Plasmids used in this study

Plasmid	Description	Source
YCplac22	*TRP1*, yeast centromeric plasmid, amp^R	Gietz and Sugino (1988)
YEplac112	*TRP1*, yeast episomal plasmid, amp^R	Gietz and Sugino (1988)
YIplac211	*URA3*, yeast integrating plasmid, amp^R	Gietz and Sugino (1988)
pBluescript II KS-	Bacterial cloning vector, amp^R	Stratagene
pK18 and pK19	bacterial cloning vectors derived from pUC18/19, kan^R replaces amp^R	Pridmore (1987)
TA-cloning vector	PCR product cloning vector, kan^R	Invitrogen
pGEM-T vector	PCR product cloning vector, amp^R	Promega
pGEM7Zf(+)	Bacterial cloning vector, amp^R	Promega
pHR69	pUC19 carrying a 1.7 kb *Hin*dIII fragment of *PMR1* into which *URA3* was inserted, amp^R	Rudolph *et al.* (1989)

Plasmid	Description	Source
YCpGALFSABL	*TRP1*, yeast centromeric plasmid. Carries a streptavidin β-lactamase fusion, under *GAL1/10* promoter, amp^R	G. Stewert University Edinburgh
pGA1070	carries part of *KEX2* gene disrupted with *LEU2*	G. Ammerer University Vienna
pYJS50	*LEU2*, yeast episomal plasmid carryies a *MFα-bla* gene fusion	A. Boyd
pKpraSH	Source of protein A (*spa*) DNA, amp^R	N. Bryant
pKS*PMR1*prA	pBluescript carrying the 3'end of *PMR1* from the *Sst*II site fused to *spa*	This study
pSpaM	YCplac22 carrying the *PMR1* gene (including its promter) fused to *spa*	This study
pYCpSTREP/ pYEpSTREP	YCplac22 and YEplac112 carrying *PMR1* tagged with Strep tag at its 3' end	This study
pNB64	pK19 carrying the *KEX2* gene cloned into *Bam*HI and *Kpn*I sites	N. Bryant (1992) (University Oregon

Plasmid	Description	Source
pSpaK (pNB66)	YCplac22 carrying a *KEX2-spa* gene fusion in its *Bam*HI site	Bryant (1992)
YCplac22*KEX2*	YCplac22 carrying the 3.6 kb *Kpn*I/*Bam*HI fragment from pNB64	This study
pSpaKP	YCplac22 carrying a KEX2-spa-PMA1 gene fusion	This study
pCXJKan-1	A *K. lactis* episomal plasmid, *URA3*, pKD1, amp^R	M. Stark (Chen *et al.*, 1989)
pKRIB*LAC4*del*Xma*I	source of *K. lactis LAC4* promoter	
pKla102	source of *ORF2* DNA	A. Boyd
pK18[*Eco*RI]	pK18 with its polylinker *Eco*RI site filled in	This study
pDP290	source of human elafin DNA	D. Pioli, Zeneca
pDP316	*LEU2*, yeast episomal expression plasmid, *MF*α promoter and *ADH1* terminator	D. Pioli
pAC1	*K. lactis* expression plasmid, *URA3*, pKD1, *LAC4* promoter, *ORF2* signal sequence, *ADH1* terminator, amp^R	This study
pAC2	pAC1 containing human elafin cloned into the *Stu*I site	This study

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